CHEMICAL INVESTIGATION OF IRRADIATED MEDICINAL PLANT (ANDROGRAPHIS PANICULATA)

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(RADIOLOGICAL SCIENCE)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2004

ISBN 974-04-5017-2

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was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Radiological Science)
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ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to:

- Assoc. Prof. **Malulee Tuntawiroon**, my advisor and Assoc. Prof. **Nopamon Sritongkul**, my co-advisor for their kindness and helpful suggestion throughout the study until this thesis was completed.
- Khun **Atthagowit Sanguansud**, radiological science expert for being an external examiner.
- Khun **Theerawut Pinthong**, my supervisor at the Medicinal Plant Research Institute for all facilities provided at the Pharmacokinetics Laboratory of the Department of Medical Science, Ministry of Public Health.
- Khun **Suvimol** , authorized officer of the Office of Atomic for Peace for providing facilities for sample irradiation.
- Dr. **Wanida Naklada** for her valuable guidance and suggestion in designing the laboratory structure and generous advice.
- Khun **Siriruk Nukcharoen**, secretary of the M.Sc. program of the Section of Nuclear Medicine for her warm co-operation and advice about the preparation of the thesis.
- Khun **Veerayuth Gleinglumyong**, member of Pharmacokinetics Laboratory for his help in performing laboratory experiments.
- **My family**, especially my parents and my friends for their help, understanding and encouragement throughout the my long days of study and preparation of the thesis.

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CHEMICAL INVESTIGATION OF THE IRRADIATED MEDICINAL PLANT *Andrographis paniculata*.

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ABSTARCT

Andrographis paniculata has been used for centuries in Asia to treat GI (Gastro-Intestinal) tract and upper respiratory infections, fevers, and chronic and infectious diseases. This paper studied the extraction technology of an active ingredient from A. paniculata by thin layer chromatography and HPLC (High Performance Liquid Chromatography), and investigated the alteration of the active constituent (andrographolide) after treatment with Co-60 irradiation to destroy microorganisms that cause spoilage and decomposition. Furthermore, changes in the contents of andrographolide after an irradiation dose of 5, 10 and 25 kGy were examined and compared with the control samples throughout a 50 day storage period. The active compound was identified by co-chromatography with a reference standard on TLC (Thin Layer Chromatography). Quantitative analysis was undertaken by HPLC with UV detection wavelength at 228 nm. HPLC was shown to be a suitable method with respect to separation efficiency and repeatability. Good separation was achieved using chloroform extract with a mobile phase containing acetonitrile and 0.1% phosphoric acid in water. The yield of crude andrographolide isolated was found to be 1.43 ± 0.02%, 1.52 ± 0.02% and 2.17 ± 0.06% for sample A, B and C respectively. The coefficient of variation (%CV) was between 0.63% and 2.57%. Irradiation doses of 5, 10 and 25 kGy did not exhibit any difference in the content of andrographolide as compared to the control samples. The stability of andrographolide was found to be highly stable over the period of 50 days. No significant change in the content was observed. It could be concluded that the content of andrographolide was not affected either by an irradiation dose up to 25 kGy or storage period up to 50 days.

KEY WORDS: ANDROGRAPHOLIDE / DITERPENE LACTONE / KALMEGH

73 pp. ISBN: 974-04-5017-2

การศึกษาคุณลักษณะทางเคมีของพืชสมุนไพรฟ้าทะลายโจรที่ผ่านการฉายรังสี (CHEMICAL INVESTIGATION OF IRRADIATED MEDICINAL PLANT ;Andrographis paniculata)

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บทคัดย่อ

ฟ้าทะลายโจรเป็นสมุนไพรที่มีการใช้อย่างแพร่หลายในหลายประเทศแถบเอเชียในรูปยารักษาโรคเพื่อบรรเทาอาการไข้ เป็นหวัด แก้เจ็บคอ, แก้ท้องเสีย, ท้องร่วง, รักษาโรคติดเชื้อของระบบทางเดินอาหาร และระบบทางเดินหายใจ งานวิจัยชิ้นนี้ได้ศึกษาวิธีการสกัดสารสำคัญที่ชื่อ “ แอนโดรกราฟอไลด์ ” ที่มีในสมุนไพรฟ้าทะลายโจรโดยใช้เทคนิคโครมาโทรกราฟี คือ Thin Layer Chromatography (TLC) และ High Performance Liquid Chromatography (HPLC) เพื่อต้องการตรวจสอบการเปลี่ยนแปลงของปริมาณสารสำคัญแอนโดรกราฟอไลด์ก่อนและหลังการฉายรังสีแกมม่าจากโคบอลต์-60 เพื่อยืดอายุในการเก็บรักษาและทำให้ปลอดเชื้อซึ่งเป็นสาเหตุหนึ่งที่ทำให้เกิดความเสียหายและเกิดการเสื่อมสภาพของอาหารหรือยาได้ จึงได้ทำการตรวจสอบการเปลี่ยนแปลงของปริมาณสารแอนโดรกราฟอไลด์ภายหลังการฉายรังสีที่ปริมาณรังสีเท่ากับ 5, 10 และ 25 กิโลเกรย์ เพื่อเปรียบเทียบกับกลุ่มควบคุมที่ไม่ได้ฉายรังสีในระยะเวลาการเก็บรักษา 50 วัน การยืนยันเอกลักษณ์ของสารแอนโดรกราฟอไลด์ทำโดยเทียบเคียงกับสารมาตรฐานของแอนโดรกราฟอไลด์ด้วย TLC และหาปริมาณโดย HPLC เนื่องจากเป็นวิธีที่มีความเหมาะสมให้ประสิทธิภาพในการแยกสารได้สูง และยังมีความถูกต้องสูง โดยทำการตรวจวัดการดูดกลืนคลื่นแสงอัลตร้าไวโอเลทที่ความยาวคลื่น 228 นาโนเมตร

สารสำคัญแอนโดรกราฟอไลด์อยู่ในส่วนของการสกัดด้วยคลอโรฟอร์ม และเฟสเคลื่อนที่ที่ใช้ใน HPLC คือ อะซีโตไนไทรและกรดฟอสฟอริคเข้มข้น 0.1% ในน้ำ ปริมาณร้อยละของสารแอนโดรกราฟอไลด์ที่สกัดได้จากสมุนไพรฟ้าทะลายโจรกลุ่ม A, B และ C มีค่าประมาณ 1.43 $\pm$ 0.02%, 1.52 $\pm$ 0.02% และ 2.17 $\pm$ 0.06% ตามลำดับ และสัมประสิทธิ์ความแปรปรวนมีค่าระหว่าง 0.63% ถึง 2.57% จากการทดลองได้ผลว่าปริมาณของสารแอนโดรกราฟอไลด์ในกลุ่มที่ฉายรังสีที่ปริมาณรังสี 5, 10 และ 25 กิโลเกรย์ ไม่มีความแตกต่างกับกลุ่มควบคุมที่ไม่ได้ฉายรังสีเนื่องจากโครงสร้างโมเลกุลของสารแอนโดรกราฟอไลด์มีความเสถียรสูง ดังนั้นจึงสรุปได้ว่าปริมาณรังสีที่สูงถึง 25 กิโลเกรย์และในระยะเวลาการทดสอบ 50 วัน ไม่ส่งผลกระทบและ ไม่พบความเปลี่ยนแปลงอย่างมีนัยสำคัญของปริมาณสารแอนโดรกราฟอไลด์.

73 หน้า ISBN: 974-04-5017-2

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LIST OF ABBREVIATIONS

Abbreviations	Term
°C	degree of Celsius
cm	centimeter
CD4(+)	T-lymphocytes
Co-60	cobalt-60
CV	coefficient of variation
DHT	dihydrotestosterone
e-beam	electron beam
eV	electron volt
gm	gram
GC	gas chromatography
Gy	gray
HIV	human immuno-defficiency virus
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
IR	infrared
k'	capacity factor
kGy	kiloGray
kg	kilogram
kV	kilovoltage
MEKC	micellar electrokinetic capillary chromatography
mg	milligram
ml	milliliter
MS	mass spectrophotometry
nm	nanometer
nM	nanomolar

LIST OF ABBREVIATIONS (continue)

Abbreviations	Term
N_m	number of molecules of solute in mobile Phase
N_s	number of molecules of solute in stationary Phase
NMR	nuclear magnetic resonance spectroscopy
ODS	octadacylsilane
PAF	platelet activating factor
pH	acidic level
RPC	reversed phase chromatography
SD	standard of deviation
SGOT	Serum glutamic oxaloacetic transaminase enzyme
SGTP	gluthatione s-transferase
TBHP	tert-butylhydroperoxide
TLC	thin layer chromatography
t_m	dead time
t_R	retention time
UV	ultra-violet radiation
V_R	retention volumn
μm	micrometer

CHAPTER 1
INTRODUCTION

The use of plants as medicine is of interest in medical practice for thousands of years. Every culture on the planet has a tradition of herbal knowledge. In general, herbs can treat a wide variety of diseases and condition. Herbal medicine is a very good alternative for those who are looking for a natural alternative for the conventional Western medicine.

Andrographis paniculata (Burm f.) Nees (AP) also known commonly as "King of Bitters," is a member of the plant family Acanthaceae. It is widely distributed in China, India, Thailand, Vietnam and Indonesia. *Andrographis paniculata* has been used for centuries in Asia to treat GI tract and upper respiratory infections, fever, herpes, sore throat, and a variety of other chronic and infectious diseases. The whole plant is used in medicine (1, 2, 3).

Andrographis paniculata has long been used in both traditional Chinese medicine and ayurvedic medicine. The major ingredients in *Andrographis paniculata* are compounds known as andrographolide, which are believed to reduce inflammation and boost the immune system. The leaves and flowers are used medicinally. Most commonly, it has been used for digestive problems and various infections.

After cultivation, *Andrographis paniculata* is generally available as dried herb powder. Unfortunately, it is often contaminated with high levels of bacteria, molds and yeasts, if untreated, the herbs may result in destroying its active ingredients. Food irradiation is a mean of preservation, it is used to extend the product shelf-life. However, the radiation energy used may cause changes in molecular structure.

In this study, we report a simple method for isolation of andrographolide from the leaves of *Andrographis paniculata.* The compound was identified by TLC and quantitative analysis by HPLC.

Moreover any changes in the content of active compounds after irradiation under different dosage from 5 to 25 kGy was investigated and compared with the controls for the period of 50 days after irradiation.

CHAPTER 2
PRINCIPLE AND REVIEW

1. Review of Andrographis paniculata

Family: Acanthaceae incidentally, the genus Andrographis consists of 28 species of small annual shrubs essentially distributed in tropical Asia. Only a few species are medicinal, of which A. paniculata is the most popular (2).

(a) (b) (c)

Figure 2.1 Shows the pictures of *Andrographis paniculata* (a) The part of leaves of *Andrographis paniculata* , (b) The whole plant body and (c) The part of flower

Distribution: Kalmegh is common name of *Andrographis paniculata* an annual herb found through India, China and Asia , specially in dense forests and moist shady places. It is under cultivation in many country of Asia and Southeast Asia. It has often been called "Indian Echinacea".

Medicinal Properties: *Andrographis paniculata* is a traditional Indian herb used for support healthy immune system. Kalmegh is also a reputed homoeopathic drug

Andrographis paniculata is prepared from fresh leaves and is given to children suffering from stomach complaints. Recent experimental finding indicated that Kalmegh is having anti-typhoid and antibiotic properties (1).

Chemical Constituents: The main compound found in *A. paniculata* is a diterpene lactone called andrographolide B, as there are several different types of andrographolide but differ amounts of harvest time, method and the parts of plant. The structure of andrographolide is shown in Figure 2.2

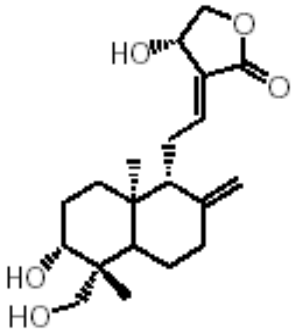


Figure 2.2 The chemical structure of Andrographolide

Scientific Evidence :

The most common therapeutic potential of *Andrographis paniculata* is its liver protective property. Its liver protective effects by enhancing activity of antioxidant enzymes along with the level of glutathione and decreasing the activity of lipid peroxidase which leads to generation of free radicals damaging the liver cells thus exerting a hepatoprotective activity.

Andrographolide have potential enhances of stimulate immune system. Some pharmacological effects are as follows:-

- Abortifacient (abortion activity)
- Analgesic (pain relief)
- Anti-inflammatory activity
- Antibacterial activity
- Antiviral activity
- Anticancer activity

- Cardioprotective (protects heart muscles)
- Choleretic (alters the properties of bile)
- Digestive enhancing
- Hepatoprotective activity
- Hypoglycemic (blood sugar reducer)
- Immune Enhancement
- Thrombolytic problem

Mechanisms of Action :

Andrographis paniculata has been extensive studied on pharmacological composition, safety, efficacy, and mechanisms of action. In the base of all cells have contain receptors when a molecule binds to the receptor, a chemical message is transmitted to targets in the cell or to other molecules in the cell, which carry the message further. The message will eventually reach the nucleus of the cell where the genetic material is stored. The DNA will be activated and the cell will respond according to what type of cell it is.

Andrographolide is a potent antiviral agent and is also anti-proliferateon by releasing interferon and activity of the lymph system. It is more effective when combined with immune stimulators, such as zinc and vitamin C. The lymph system is an important part of the immune system, carries away the by-products of cellular metabolism and also acts as a shuttle for invading bacteria and viruses, taking them to the lymph nodes where the white blood cells (lymphocytes) destroy them. Andrographolide may also be useful in cancer therapy.

Biodistribution

Biodistribution experiments have been done in experimental animals. Following injection of radioactively labeled andrographolide, this compound appears to be widely distributed in the body. High concentrations are noted in the central nervous system (brain and spinal cord) and other organs with high blood flow, including the colon, spleen, heart, lungs, and kidneys.

Biological half-life

Andrographolide appears to have a relatively short half-life of approximately two hours. Compounds with short half-lives need to be given often since they do not stay in the body for long. Andrographolides are excreted fairly rapidly from the body via the urine and gastrointestinal tract. In some studies, 80 percent of the administered dose of andrographolide is removed from the body within eight hours, with excretion rates of more than 90 percent of the compound within forty-eight hours (1).

Cultivation:

In India, it is cultivated as rainy season. It likes high moisture and can grow in any soil having organic matter is suitable for cultivation. About 400 gms. seed are sufficient for one hectare. The spacing is maintained 30 × 15 cm. No major insect and disease infestation has been reported. The plants at flowering stage 90–120 days after sowing. About 50–60 days after first harvest, final harvest is performed.

Medical Research:

Myocardial infarction

The published studies from China indicate the extracts of the herb have successfully inhibited the thickening of arterial walls following experimental angioplasty and damage to the heart following induced myocardial infarctions in experimental animals. Separate studies show that Andrographolide inhibits aortic smooth muscle cell proliferation in vitro. The potential exists for use of Andrographolide in many types of patients with cardiac risk (3).

Hepatoprotective effects

Toxicologists are currently studying Andrographolide for its hepatoprotective effects. Visen and co-workers compared the hepatoprotective effects of silymarin and Andrographolide on rat hepatocytes exposed to paracetamol. Andrographolide was remarkably superior in protecting the viability of hepatocytes and in reversing the toxic effects of paracetamol on certain enzymes (eg. SGOT, SGTP, alkaline phosphatase) in serum (4).

Kapil and co-workers studied the hepatoprotective effects of Andrographolide

against carbon tetrachloride and tBHP intoxication. Andrographolide was shown to be equvalently effective as silymarin with respect to its effects on the formation of the degradation products of lipid peroxidation and the release of glutamic-pyruvate transaminate and alkaline phosphatase in the serum (5).

Andrographolide was found to be more potent than silymarin (standard hepatoprotective agent) . It protects rat hepatocytes against paracetamol-induced toxicity. Protective effects on hepatotoxicity induced by carbontetrachloride. The 5 parameters GOT, GPT, serum alkaline phosphatase, serum biliburin and hepatic triglycerides are increase cause of inhibition 48.6 % andrographolide, 32.0% methanolic extract and 15% andrographolide-free methanolic extract (5, 6, 7).

Immunostimulating properties

Andrographolide also exhibits immunostimulating properties. Puri and co-workers found that Andrographolide extract induced significant stimulation of antibody and delayed hypersensitivity response to sheep red blood cells. The plant preparation also stimulated nonspecific immune responses as measured by macrophage migration index, phagocytosis of E. coli and proliferation of spleenic lymphocytes (8).

Andrographolide produces significant dose-dependant choleretic effects (4.8 - 73%) as evidenced by increase in bile flow, bile salts, and bile acids. Andrographolide was found to be more potent than silymarin, a clinically used hepatoprotective agent (9).

Fever relief

In another study by Thamlikitkul's research team, 152 adult patients with pharyngotonsilitis were enrolled in a randomized double-blind study to assess the efficacy of Andrographis paniculata. The patients were randomized to receive either paracetamol or 3 g/day of Andrographis paniculata or 6 g/day of Andrographis paniculata for 7 days. The efficacy of paracetamol or high dose andrographolide was significantly more than that of low dose andrographolide at day 3 in terms of relief of

fever and sore throat. The same clinical improvement was noted on day seven (10).

Inhibiting cancer cells

Andrographolide has been shown to inhibit human breast, liver, and prostate cancer cells. In tests conducted in China on prostate cancer cell lines, it was found to be as effective as the cytotoxic drug, cisplatin. Oncologists at the Rosell Park Cancer Institute are currently studying the potential use of andrographolide as a therapeutic agent in several prostate cancer cell lines. Unlike cytotoxic anticancer drugs, andrographolide is rapidly metabolized when taken orally (11).

Side effects

Historical use of the crude herb, Chuanazinlian, from which andrographolide is isolated, is non-toxic in humans and does not cause the side effects of conventional chemotherapeutic agents, such as hair loss and mouth sores.

Anti HIV properties

Andrographolide esters have been found to be inhibitors against the HIV virus in vitro. It was non-toxic to the H9 cell at concentrations of 1.6 - 3.1 mcg/ml. It was also inhibitory to two other strains of HIV-1 and a strain of HIV-2 (12).

Andrographolide administered at 5, 10 and 20 mg/kg bodyweight for 3 weeks for each. It have been found significant rise in the mean of CD4(+) lymphocyte level of HIV subject andrographolide may inhibit HIV- induced cell cycle dysregulation leading to a rise in CD4(+) lymphocyte levels (13).

Other properties

Considering extensive reports of clinical efficacy in preventing restenosis in angioplasty patients and its role in the treatment of various malignancies, andrographolide may become a welcome addition to the physician's armamentarium of non-toxic therapeutic agents.

Use of *Andrographis paniculata* analysis the fertility test and histopathological by counting the results showed that the spermatozoa were not motile and several of them possessed abnormalities. Andrographis paniculata could effect spermatogenesis by preventing cytokinesis of the dividing cell lines.

The effect of andrographolide in rat by pre-oral administration during 19 days of pregnancy in doses of 200, 600 and 2000 mg/kg does not exhibit any effect on the level of progesterone in the blood plasma of rats. The extract from Andrographis paniculata cannot induce progesterone-mediated termination of pregnancy (14).

The possible testicular toxicity of *Andrographis paniculata*, standardized dried extract was evaluated in male Sprague Dawley rats for 60 days. No testicular toxicity was found with the treatment of 20, 200 and 1000 mg/kg during 60 days as evaluated by reproductive organ weight, testicular histology, ultrastructural analysis of Leydig cells and testosterone levels after 60 days of treatment. It is concluded that Andrographis paniculata dried extract did not produce subchronic testicular toxicity effect in male rats (15).

Isolation Method:

The isolation of andrographolide from the leaves of *Andrographis paniculata* is reported. This involves of the leaf powder by cold maceration in a 1:1 mixture of dichloromethane and methanol and extract by recrystallisation (16). Identified the compound by inspected through IR, NMR, mass and melting point, and chromatography. The purity of the compound was confirmed by UV absorption spectrum, HPLC and differential scanning calorimetry, the latter of which gave the melting point of andrographolide as 235.3 ^{o}C.

High-performance thin-layer chromatographic (HPTLC) method has been developed for the simultaneous quantitative estimation of the biologically active diterpenoids, 14-deoxy-11,12-didehydroandrographolide, andrographolide, neoandrographolide and andrographiside in *Andrographis paniculata*. The assay combines the isolation and separation of andrographolide derivatives on silica gel 60

F_{254} HPTLC plates with spot visualization and scanning at 540 nm. Methanol was found to be the most appropriate solvent for the exhaustive extraction of andrographolide derivatives (17).

A simple method for analysis of andrographolide in the serum of rats by HPLC and photodiode array detection was developed. Andrographolide a diterpene lactone was extracted with acetone (4:1) and then analyzed using a RP-C18 lichrospher reversed phase column (4x125mm). A mobile phase consisting of 26% acetonitrile and 0.5% of phosphoric acid, at a flow rate of 1.1 mL/min, at 228 nm was used (18).

2. **Review of Chromatography**[19-25]

2.1 Definition of chromatography [19,20]

Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system which distributed between two phases.

Liquid chromatography (LC), is chromatographic procedure in which the mobile phase is a liquid. High performance liquid chromatography is one of the high efficiency chromatographic techniques with less errors in quantitation. Variously known as high resolution, high pressure, high performance and high speed referred to as HPLC. HPLC is used for a wide range of applications and offers significant advantages in the analysis of pharmaceutical formulations, biological fluid, a variety of inorganic substances and trace element contaminations.

2.2 Types of Chromatography

There are many types of chromatography but all are based on the principle that the **mobile phase** carries the compounds to be separated and a **stationary phase** binds these compounds through intermolecular forces (Table 2.1). The principles are as follows:

- **Adsorption chromatography** uses a solid stationary phase and a liquid or gaseous mobile phase. Solute is adsorbed on the surface of the solid particles.

- **Partition chromatography** involves a thin liquid stationary phase coated on the surface of the solid support. Solute equilibrates between the stationary liquid and the mobile phase.
- **Ion-exchange chromatography** features ionic groups such as $-SO_3^-$ or $-N(CH_3)_3^+$ covalently attached to the stationary solid phase. Solute ions are attracted to the stationary phase by electrostatic forces. The mobile phase is a liquid.
- **Molecular exclusion chromatography** separates molecules by size, with larger molecules passing through most quickly. The stationary phase has pores small enough to exclude large molecules but not small ones.
- **Affinity chromatography** employs specific interactions between one kind of solute molecule and a second molecule that is covalently attached to the stationary phase.

Applications of HPLC partition chromatography are divided into categories (21,23,24)

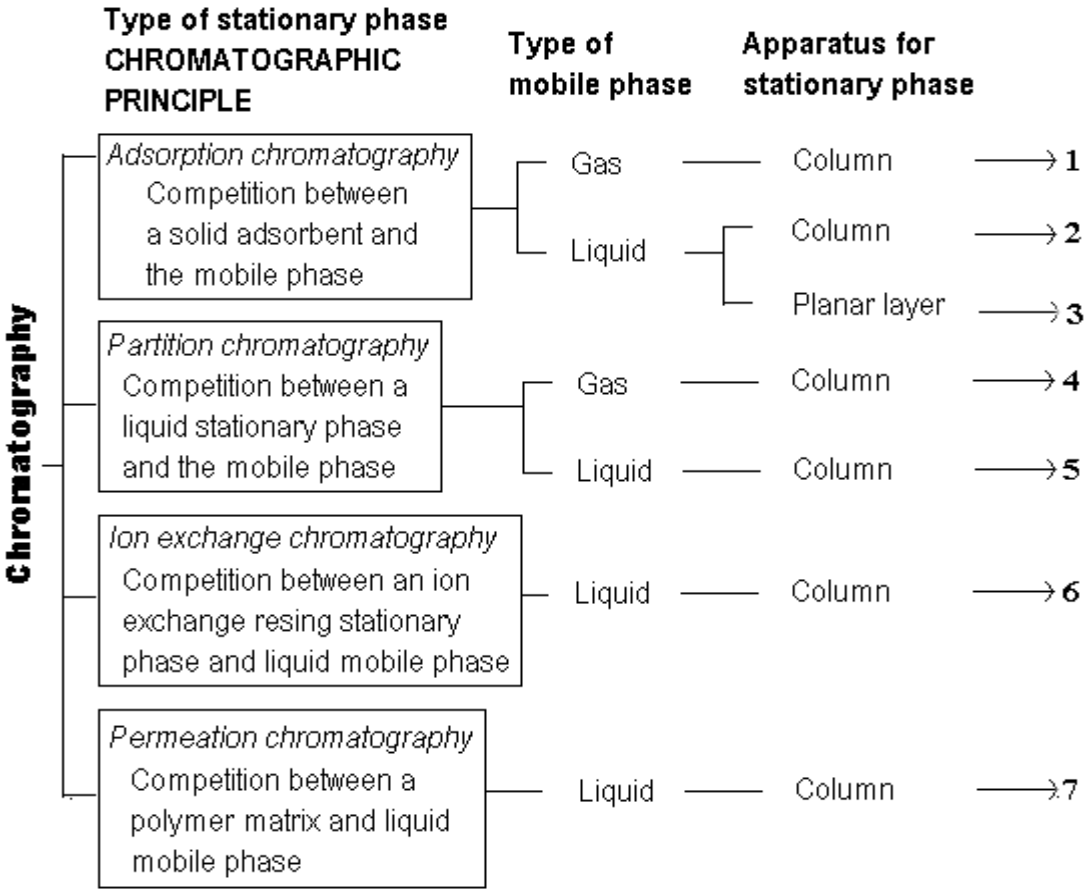
1. Liquid-liquid chromatography - the packing material is coated with a non-covalently bound material adsorbed on the surface.
2. Liquid-bonded phase chromatography - the stationary phase liquid coating is covalently bonded.
3. Liquid-solid chromatography - the stationary phase is a polar solid surface (e.g., silica $(SiO_2)_n$), alumina $(Al_2O_3)_x$, charcoal, clay, other).

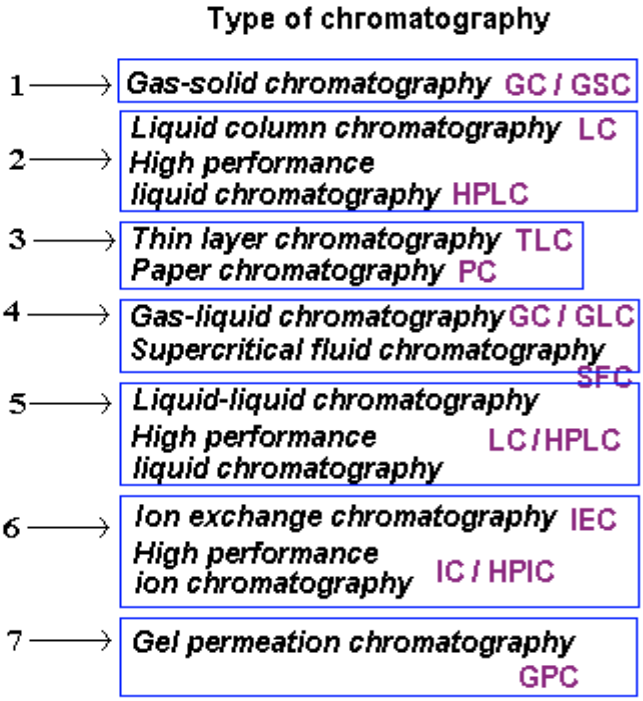
Liquid-liquid and liquid-bonded chromatography may be run as

1. Normal phase chromatography - partition chromatography where the stationary phase is polar and the mobile phase is nonpolar.
   a. The least polar components elute first.
   b. Increasing polarity of the mobile phase decreases elution time.
2. Reverse phase chromatography - partition chromatography where the stationary phase is non-polar and the mobile phase is polar.
   a. The most polar components elute first.
   b. Increased polarity of mobile phase increases elution time.
   c. Most popular HPLC about 75% was reverse phase and mobile phase was

used water and methanol.

Table 2.1 Chart of typical chromatography





The limitations of classical liquid liquid partition chromatography (LLC) systems led to the development of packing material were the stationary phase is chemical bonded or organo bonded to an insoluble matrix. The most stationary phase is octadecylsilane (ODS), which is bonded to a silica support via a silyl ether (siloxane) linkage (Figure 2.3). Packing material is used with a polar eluent, e.g.methanol or water, an elution technique commonly referred to as reverse phase chromatography.

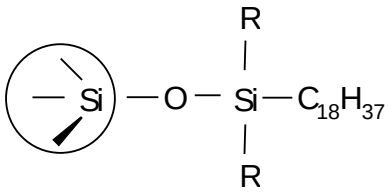


Figure 2.3 Octadacylsilane (ODS) chemically bonded stationary phases.

The range of stationary phases, though extremely varied in character, is small in number due to the expensive control that can be exerted over selectivity by variation in the eluent composition, a feature in marked contrast to GC(25).

2.3 Partition Chromatography

2.3.1. The separation mechanism

Reversed Phase Chromatography (RPC) utilizes solubility properties of the sample. The distribution of sample components between the two phases will depend on their respective solubility characteristics and the polar properties of the phases.

In the column the sample is continuously re-partitioned between a stationary non-polar phase (the beads in the column) and a mobile phase (the liquid pumped through the column).

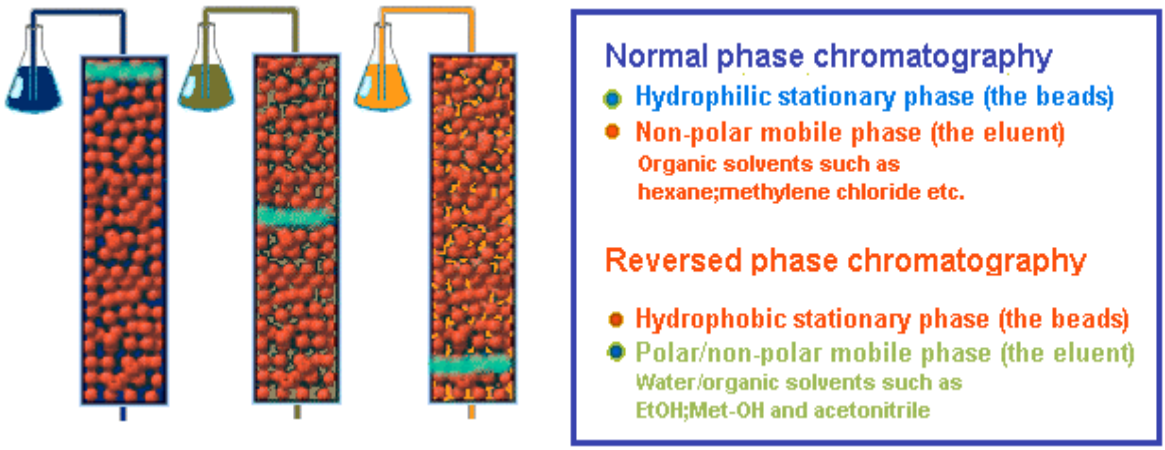


Figure 2.4 The distribution between the stationary and the mobile phase is controlled by the polar properties of the mobile phase.

The partition of solutes between two liquid phases have differences in polarity or the covalent bonding of the stationary phase molecules to a solid support. The latter system includes a widely used reversed phase procedure in which a solid support phase such as silica is extensively reacted with mono-, di-, or triorganochlorosilanes which cover the polar surface with long C8 to C18 hydrocarbon chains.

The principle of the method is illustrated schematically in Figure 2.5.

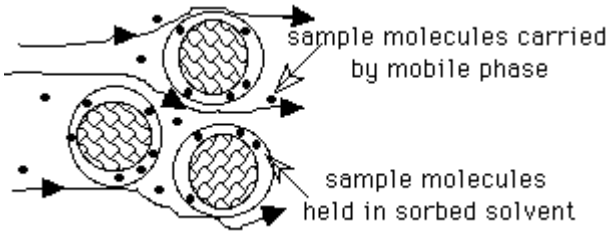


Figure 2.5 Stationary phase = sorbed solvent held on the surface, or within the grains or fibers of an inert solid supporting matrix

Sample molecules equilibrate (partition) between liquid stationary phase and mobile phase.

2.3.2. Solubility properties

The ability of a solvent to dissolve a substance depends on its ability to interact with the substance. Non-polar substances are held together by Van der Waals interactions (Figure 2.6) which polar solvents do not break, since dipole-dipole interaction between the solvent molecules is too strong.

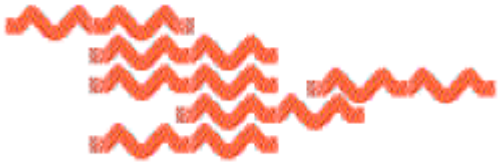


Figure 2.6 Hydrocarbon chains attract each other mainly by Van der Waals interactions.

Polar substances are held together by the attraction between dipoles (Figure 2.7). It takes polar solvents to break these bond in order to bring about solubilisation.

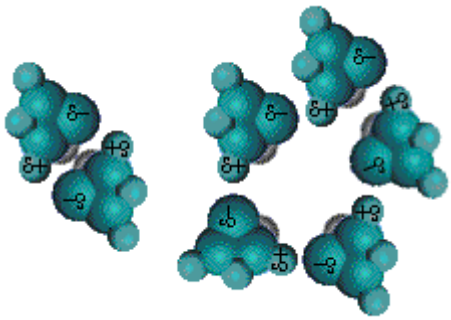


Figure 2.7 Dipoles interact in a head-to-tail manner between the polar centres.

Hydrogen bonding is typical for water and makes water molecules appear in clusters rather than as "free" single molecules (Figure 2.8). The HX- group is also a dipole, which makes water an excellent solvent for polar substances especially those containing HX groups (X= O; N; F; Cl)

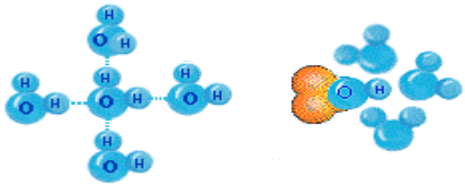


Figure 2.8 Hydrogen bonding occurs between molecules containing HX-groups.

2.3.3. Adsorption mechanisms

Reversed phase chromatography; RPC was first applied to relatively small organic molecules which more or less dissolved in the hydrocarbon phase. For example peptides and proteins carry a mix of hydrophilic and hydrophobic amino acids accessible for interaction with the RPC ligands. They are rather large at least in comparison to the traditional organic target molecule. They therefore cannot be completely "embraced" by the hydrocarbon phase. Instead there is a high probability for multi-point attachment. Hydrophobic surfaces are known to combine by a mechanism called hydrophobic interaction also shown in Figure 2.9 and 2.10.

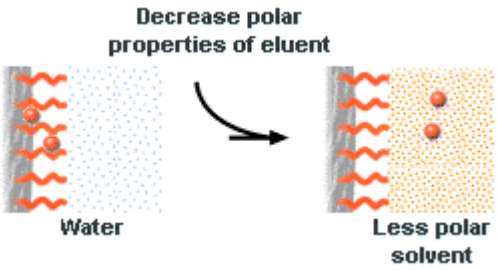


Figure 2.9 Organic molecules are "embraced" by the carbon chains of the stationary phase

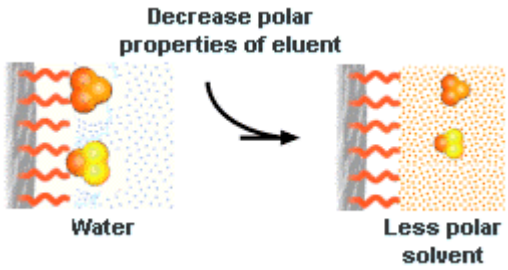


Figure 2.10 Typical organic target molecule, peptides and protein adsorb to the stationary phase.

2.3.4. Desorption curves

The adsorption reaction is a dynamic equilibrium between free and adsorbed molecules and is controlled by the content of an organic solvent in the eluent. It can be

described in terms of a desorption curve obtained by plotting the relative amount of free sample molecules as a function of the organic solvent concentration as shown in Figure 2.11 (Desorption curves have little practical value and are used here only to demonstrate the working principles of RPC.)

The desorption curve thus represents the velocity of a sample zone as a function of the organic solvent concentration. The concentration interval corresponding to the desorption curve will be referred to as the partition zone.

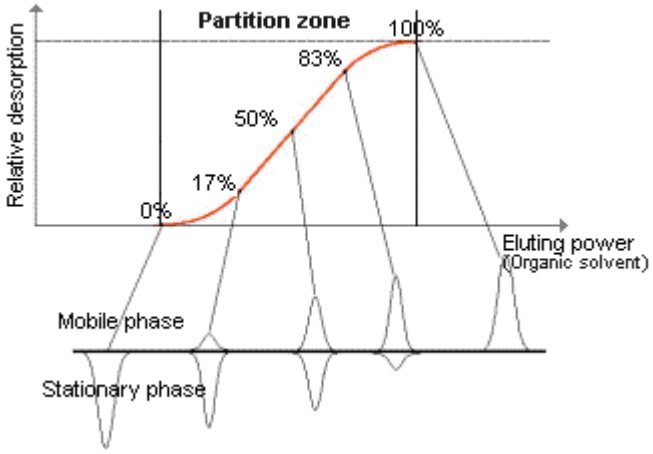


Figure 2.11 The desorption curve reflects the distribution of the sample between the mobile and the stationary phase.

Retention depends on a sample molecule's escaping into the mobile phase versus its solubility in the stationary phase.

Quantitatively given by the partition coefficient, K_D, the ratio of solubility in the two phases

$$K_D = \frac{\text{[solute in mobile phase]}}{\text{[solute in stationary phase}}$$

2.3.5. Elution modes

With many modes of chromatography the performance can be significantly improved by altering the characteristics of the mobile phase during the chromatographic process. Normally the composition of the mobile phase entering the

column is held constant and this procedure is referred to as isocratic elution chromatography. Additional resolving power can often be achieved by a gradient elution process in which the composition of the mobile phase is programmed to change with time.

Isocratic elution

In isocratic elution the chromatographic conditions are kept constant throughout the entire experiment. This makes the basic construction of an isocratic chromatography system rather simple. The isocratic experiment works within the partition zones for the sample components to be separated.

The longer column will always provide better resolution when run under isocratic conditions. However, one need to increase the column length four times to double the resolution.

Gradient elution

The most frequently used elution mode in high resolution protein/peptide applications of RPC is gradient elution. Gradients of organic solvents are formed by mixing two eluents, one containing no or a low concentration of the organic solvent (buffer A) and one containing a high concentration of this solvent in water (buffer B). But for their organic solvent contents, the two eluents are identical. Chromatography systems usually control the gradient formation by the use of two pumps, one for buffer A and one for buffer B.

2.4 Resolution in RPC

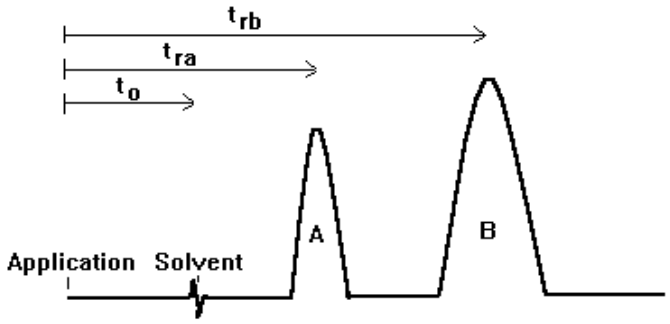
In RPC, selectivity (distance between and order of eluted peaks) depends on the hydrophobic properties of the individual sample components. With proteins and peptides the hydrophobic properties are influenced by the running pH. Ion pairing agents influence the hydrophobic properties by "blocking" either positive or negative charges and thus selectivity. Gradients will influence peak-spacing but not elution order. Efficiency (counteraction of zone broadening) depends on bead size, quality of the packed bed and flow rate in isocratic and gradient modes.

Best resolution is theoretically obtained in isocratic mode. For practical reasons, however, gradient mode is the most frequently used elution technique.

2.5 Theory

2.5.1. Principles of Chromatographic Separation

The objective of any chromatographic system is to separate components of a mixture. Liquid chromatographic systems exploit different affinities of the components for a stationary phase packed into a column and a mobile liquid phase that passes through it. Successful separation can be monitored by using an appropriate method to detect the compounds in the column effluent. An elution chromatogram usually relates the concentration of the components in the mobile phase with time from application of the mixture to the column. The principle is illustrated in Figure 2.12



Column Capacity Ratio $k = (t_r - t_o)/ t_o$

$k_a = (t_{ra} - t_o)/ t_o$

$k_b = (t_{rb} - t_o)/ t_o$

t = time or elution volume

assume a constant rate of flow.

Figure 2.12 separation of components A and B of the mixture

This shows that components A and B of the mixture applied to the column are retained on the column for different times. This is because B has a greater affinity than A for the stationary phase in the column and this represents the basis upon which the separation is achieved.

The **Column Capacity Factor**, is the ratio of the mass of the compound in the stationary phase relative to the mass of the compound in the mobile phase. The capacity factor is a unitless measure of the column's retention of a compound. This being so the ratio can be more correctly, but less conveniently, expressed in terms of retention volumes. Small values of k indicate that the components are not significantly retained by the column consequently there is poor separation of solute mixtures.

$$k' = (t_R - t_M) / t_M$$

2.5.2 Coulomb's Law (like attracts like)

Intermolecular forces which influence retardation of components are based on Coulomb's Law -like attracts like- attraction occurs between molecules with similar electrostatic properties but molecules with dissimilar properties are repelled. Electrostatic interactions between molecules are of two main types:

- polar van der Waal's retention forces arising from interaction between molecules having a surface charge;
- non-polar dispersion forces between neutral molecules or functional groups;

Ion chromatography separations are based on the strong attraction between ions of opposite charge and the exchange of ions between the analyte in the mobile phase and the stationary phase.

Hydrogen bonding between a component and the stationary phase (or mobile phase) is a relatively strong attractive force which in GC and HPLC can lead to slow equilibrium processes and tailing of peaks.

2.5.3 Retention Time

Figure 2.13 is a typical chromatogram for a sample containing a single analyte. The volume of mobile phase required to carry a band of component molecules through the system to the detector is termed the retention volume, V_R, and is measured from the start of the chromatography to the peak maxima. However, it is difficult to accurately measure volume flow rates of the mobile phase in column chromatography systems such as GC and HPLC. Therefore the time taken by a component band to pass through the column is recorded as the retention time.

The time it takes after sample injection for the analyte peak to reach the detector is called the retention time and is given the symbol t_R. The small peak on the left is for a species that is not retained by the column. The sample or the mobile phase will ordinarily contain an unretained species. When they do not, such a species may be added to aid in peak identification. The time t_M for the unretained species to reach the detector is sometimes called the dead time**.**

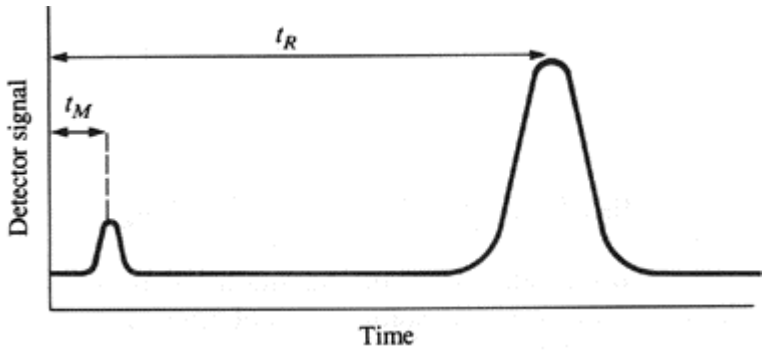


Figure 2.13 A typical chromatogram for a two component mixture.

2.5.4 Selectivity Factor

The ability of a stationary phase to separate two components A and B, where B is the more strongly retained component. The **selectivity factor** alpha of a column for the two adjacent peaks of the two species A and B in a chromatogram . The selectivity is always equal to or greater than one. If the selectivity equals one the two compounds

cannot be separated. The higher the selectivity, the more separation between two compounds or peaks.

$$\text{alpha} = k'_B / k'_A$$

where k'_B and k'_A are the capacity factors for B and A, respectively.

2.5.5 Theoretical Plates

The band broadening that occurs in column chromatography is the result of several factors, which influence the efficiency of a column.

The separation efficiency of a column can be expressed in terms of the number of theoretical plates in the column. The terms, the number of theoretical plates and plate height have their origin in the plate model of the chromatographic process. The plate model is useful for characterizing the efficiency of distillation columns and liquid extractors.

A theoretical plate is a defined definition that we can visualize from the liquid-liquid countercurrent distribution solvent extraction system. There, two phases contact each other in separate tubes and are incrementally passed along one another after equilibrium is achieved. Similarly, each theoretical plate in chromatography can be thought of as representing a single equilibrium step. In reality, they are a measure of the efficiency of a column. For high efficiency, a large number of theoretical plates is necessary. The height equivalent to a theoretical plate (HETP) is the length of a column divided by the number of theoretical plates. To avoid a long column, then, the HETP should be as short (thin or small) as possible. These definition apply to all forms of column chromatography and also to distillation separations, but the parameters are easier to determine in gas chromatography.

2.5.6 The Height Equivalent to a Theoretical Plate (HETP)

The plate model supposes that the chromatographic column is contains a large number of separate layers, called theoretical plates. Separate equilibrations of the

sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next.

$$HETP = L / n$$

where HETP is the number of theoretical plates of a column toward a particular compound.

An alternative way to estimate the number of theoretical plates is from the width of the peak measured at a height of one-half of the peak height, $w_{1/2.}$ The width of a peak, then, is related to the HETP, being narrower with a smaller HETP.

$$n = 5.55\ t_R^2 / w_{1/2}^2$$

2.5.7 Column Resolution

The **resolution;** R_S of a column provides a quantitative measurement of its ability to separate two analytes. The significance of this term is illustrated in Figure 2.14 which consists of chromatograms for species A and B on three columns with different resolving powers.

The resolution R of a column indicates the ability to separate two species (analytes).

$$R_S = 2\ (\ (t_R)_B - (t_R)_A\ )\ /\ (\ W_A + W_B\ )$$

where $W_A/2$ and $W_B/2$ are half the peak widths of species A and B respectively. δZ is the distance between (the tops of) both peaks, it can be written as $t_R(B) - t_R(A)$. Baseline resolution is achieved when R = 1.5. Retention times would be increased in direct proportion to the length of the column.

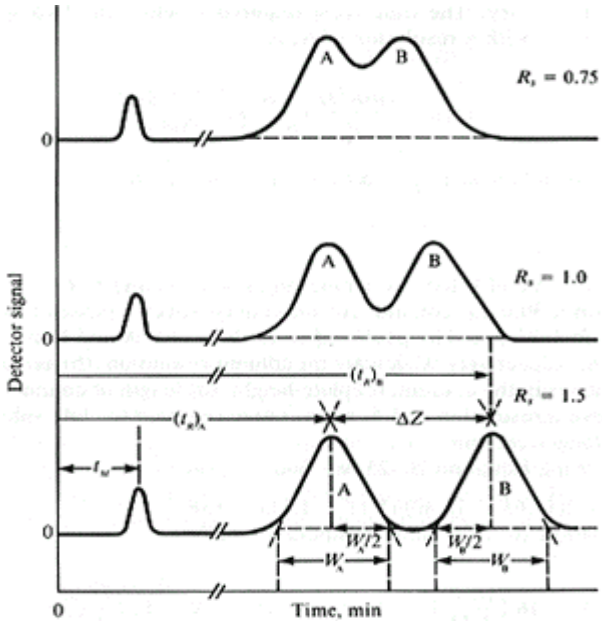


Figure 2.14 Column Resolutions of Chromatographic Separation

2.6 Peak Asymmetry - Fronting and Tailing

The normal dispersion of component molecules as they move through the chromatographic system is represented by a bell-shaped Gaussian peak (Figure 2.15).

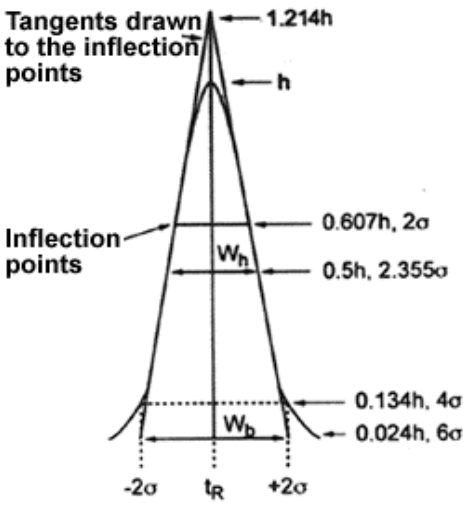


Figure 2.15 Ideal Peak: Bell-shaped Gaussian peak

However, if some molecules in the band are more strongly retained on the stationary phase due to high intermolecular forces, for example, hydrogen bonding, than predicted by the distribution ratio. K, then these molecules will lag behind the main band and will form a 'tail' on the main peak, peak tailing (Figure 2.16). Fronting occurs when some of the molecules move ahead of the main band due to less than expected retention by the stationary phase. This may be due to too large a sample being introduced onto the stationary phase such that the retention capacity is exceeded.

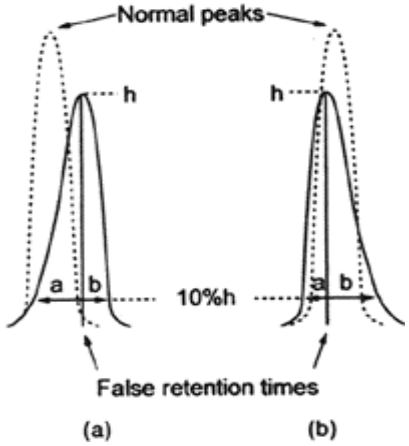


Figure 2.16 Peak asymmetry. (a) Fronting and (b) tailing.

2.7 Qualitative Analysis

Chromatography is widely used for recognizing the presence or absence of components in mixtures that contain a limited number of possible species whose identities are known. The chromatographic peaks can identified with spectrophotometer to record a complete spectrum as the substance is eluted from the column. The resulting hyphenated instruments are powerful tools for identifying the components of complex mixtures. Alternatively, eluate can be condensed and collected and subjected to any desired analysis, such as nuclear magnetic resonance.

The simplest method of identification of a chromatographic peak is comparison of its retention time with that of an authentic sample of the suspected compound. The most reliable way to do this is by co-chromatography, in which authentic sample is added to the unknown. If the added compound is identical to one component of the

unknown, the area of that one peak will increase with respect to the others in the chromatogram. It is unlikely that two compounds with the same retention time on one stationary phase will have the same retention times on two different stationary phases.

2.8 Quantitative Analysis

The quantitative analysis for measuring the quantity of components in the mixture by peak area, standard addition or the internal standard method.

Quantitative chromatography is based upon a comparison of either the height or the area of the analyte peak with that of one or more standards that the area or height of a peak is proportional to the amount of that component.

2.8.1 Analyses Based on Peak Height

The height of a chromatographic peak is obtained by connecting the baselines on the two sides of the peak by a straight line and measuring the perpendicular distance from this line to the peak but must be taken to avoid overloading the column. The effect of sample-injection rate is particularly critical for the early peaks of a chromatogram. Relative errors of 5 to 10% due to this cause are not unusual with syringe injection.

2.8.2 Analyses Based on Peak Area

Peak area is independent of broadening effects caused by the variables mentioned in the previous pages. From this standpoint, therefore, the peak area is a more satisfactory analytical parameter than peak height. On the other hand, peak heights are more easily measured and, for narrow peaks, more accurately determined. Many modern chromatographic instruments are equipped with electronic integrators that provide precise measurements of relative peak areas.

2.8.3 Calibration with Standards

The most straightforward method for quantitative chromatographic analyses involves the preparation of a series of standard solutions that approximate the composition of the unknown. Chromatograms for the standards are then obtained, and

peak heights or areas are plotted as a function of concentration. A plot of the data should yield a straight line that passes through the origin; analyses are based upon this plot.

2.8.4 The Internal Standard Method

The highest precision for quantitative chromatography is obtained by using internal standards because the uncertainties introduced by sample injection are avoided. In this procedure, a carefully measured quantity of an internal-standard substance is introduced into each standard and sample, and the ratio of analyte peak area (or height) to internal-standard peak area (or height) is the analytical parameter.

2.9 Overview of high performance liquid chromatography instrumentation

In HPLC filtered eluent is drawn from the solvent reservoirs, the eluent composition being determined by the proportion of each solvent delivered to the column via a high pressure pump and solvent mixing system. The sample mixture is applied to the top of the column and the components of the mixture are then carried out the column by the eluent. The passage of the solutes from the column is monitired by the detector and response displayed on either a chart recorder or an integrator.

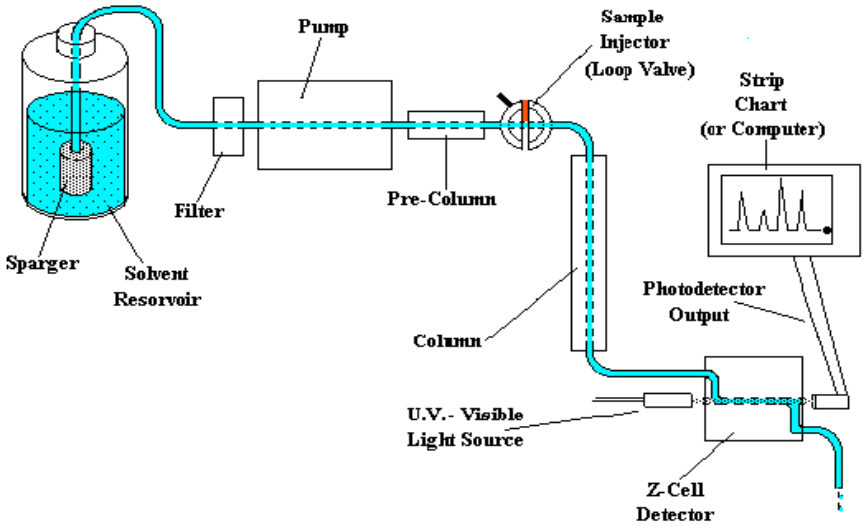


Figure 2.17 Major components and functions of the HPLC station

Major components and functions of the HPLC components:

1. One or more glass or stainless steel solvent reservoirs to hold mobile phase.
    a. Mobile phase is usually degassed via a sparger; a vacuum system, heating/stirring device may also be used.
    b. Mobile phase solutions are usually pre-filtered before use to remove dust and particulates.
    c. Elution may be:
        1) Isocratic - a constant composition mobile phase.
        2) Gradient elution - two (or more) solvents whose mix ratio is changed in a programmed way.

2. Pumping system.
    The pumping system must be:
        1) Generate pressures up to ~6,000 pounds per square inch.
        2) Give a pulse-free output (via a pulse damper).
        3) Give flow rates of ~0.1 - 10 mL/minute.
        4) High resistance corrosion .

3. Sample injection system
    a. Sample injection through rubber septum systems (like GC's) are limited to approximately 1500 pounds per square inch.
    b. Most modern HPLC's use sample injection via sampling "loops."

4. Columns
    a. Columns are usually stainless steel tubes.
    b. Lengths are typically 10 - 30 centimeters.
    c. Inside diameters of 4 - 40 mm are typical; newer high-performance columns are 1 - 4.6 mm.
    d. Particulate packing size is typically 5 - 10 micrometers.
    e. Particulate packing usually consist of small silica particles.

5. Detectors

The most common detectors are based on the visible or u.v. light absorbance of analytes using a "Z" cell.(Figure 2.21)

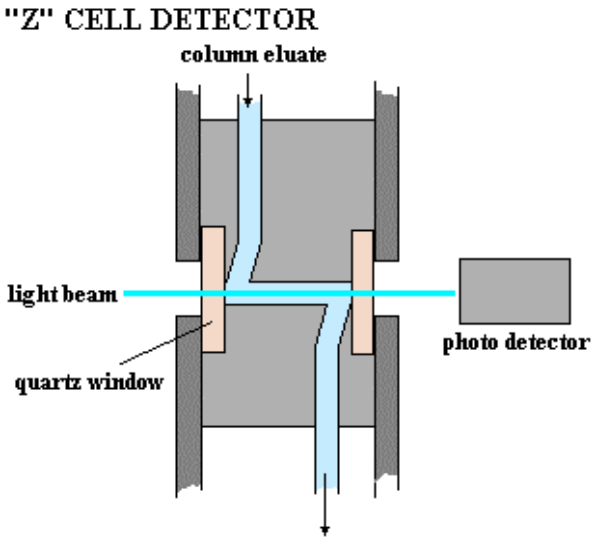


Figure 2.18 The diagram of Z Cell Detector

3. Review of Food Irradiation

Irradiation is a physical treatment of food with high-energy, ionising radiation. The main purpose of food irradiation is to destroy micro-organisms which cause food to become inedible or unsafe. It can be used to prolong the shelf life of food products and/or to reduce health hazards associated with certain products due to the presence of pathogenic micro-organisms.

The treatment may be applied for different purposes, such as:

a) Prevention of germination and sprouting of potatoes, onions and garlic.

b) Disinfection by killing or sterilizing insects which infest grains, dried fruit, vegetables or nuts.

c) Retardation of ripening and aging of fruit and vegetables.

d) Prolongation of the shelf life and prevention of food-borne diseases by reducing the number of viable micro-organisms in meat, poultry and seafood.
e) Reduction of micro-organisms in spices and herbs.

Food irradiation:

Food irradiation is a promising new food safety technology that can treat foods, either packaged. Ionizing radiation may come from one of three sources - radioactive isotopes of cobalt or cesium, electron accelerators or X-rays. All three sources produce similar effects in the short wave, high-energy region of the spectrum is treating food with ionizing radiation can kill bacteria and parasites that would otherwise cause food borne disease. Similar technology is used to sterilize medical devices and used in surgery without risk of infection.

The effects of irradiation on the food and on animals and people eating irradiated food have been studied extensively. Dangerous substances do not appear in the foods. The nutritional value of the food is essentially unchanged.

Beginning of the 21st century, foodborne disease remains a major threat to public health. Milk pasteurization and retort canning, were developed, promoted to prevention measures against foodborne diseases, as new pathogens and products have emerged (26). Many of these threats can be controlled by applying new technologies.

Dosage in food irradiation [27]

The dose is the quantity of radiation energy absorbed by the food as it passes through the radiation field during processing. Dose is generally measured in Grays (G) or kilo Grays (kGy) where 1 Gray = 0.001 kGy = 1 joule of energy absorbed per kilogram of food irradiated. Dose can also be measured in Rads (100 Rads = 1 Gray). Different dosages are used to produce different effects in foods. Some of these include: Extension of shelf life of fruits (0.5-1.5 kGy). Control of harmful bacteria in fresh meat and poultry (1.5-4.5 kGy). Control of insects, parasites or microorganisms (0.15 to <1kGy) Delay of ripening (0.5-2 kGy). Inhibition of sprouting (0.05-0.15 kGy)

Traditional Methods: Sanitation and Pasteurization

For all foods, using basic principles of sanitation and food hygiene preserves wholesomeness and shelf life. For foods susceptible to contamination with particularly deadly pathogens. , especially those pathogens that cause severe illness to humans exposed to even small amounts.

Botulism is a paralytic illness bacteria in canning food that follows ingestion of food containing botulinum toxin. Botulinum is an extremely potent toxin produced by the bacterium *Clostridium botulinum* under certain anaerobic conditions, such as those that may be found inside a hermetically sealed can. This bacterium can live inside a can because it forms a hardy spore that can survive the temperature at which water boils at ordinary air pressure. It takes higher than 100 degree Celsius temperatures to kill spores in canned food (28, 29).

Pasteurization of milk, another fundamental technology used to prevent foodborne disease. At the turn of the last century, cows' milk was recognized as the source of a large number of different infections, including typhoid fever, bovine tuberculosis, diphtheria, and severe streptococcal infections. For some, the best way to prevent infections spread through milk was to pay scrupulous attention to the health of animals and to create sanitary conditions for the milk production process.

The use of both retort canning and milk pasteurization took decades to gain universal acceptance. Many were concerned that the use of these technologies would lead to slippage of standards for quality and sanitation. These concerns were ultimately addressed by using formal grading processes to assure the public that only clean milk would be pasteurized, and only vegetables of clearly defined quality would be canned. Concerns that loss of nutrients would be an important issue were found to be unwarranted. Although a wide variety of times and temperatures were initially used, clear microbial target endpoints were ultimately defined for both canning and pasteurization so that milk pasteurization and botulism retort cook have standard meanings everywhere in the United States.

Food Irradiation Technology

The use of high energy irradiation to kill microbes in food was evaluated in this country as early as 1921, when scientists at the United States Department of Agriculture reported that it would effectively kill trichinae in pork (30). Irradiation has become a standard process used to sterilize many consumer and medical products, from adhesive strips to surgical implants. Three different technologies that can be used to treat food have been developed by the sterilization industry.

Gamma Irradiation

Gamma irradiation technology uses high energy gamma rays that are emitted by radioactive Cobalt 60 or Cesium 137. These radioactive sources are produced in commercial nuclear reactors and have a long half-life that makes them useful for commercial installation. Food or other products are brought into a heavily-shielded chamber and exposed to gamma rays for a defined length of time. When the source is not in use, it is stored in a pool of water that absorbs all irradiation, effectively turning it off. These high energy rays can penetrate deeply, making it possible to treat bulk foods on shipping pallets.

Electron Beam Irradiation

Electron beam technology uses a stream of high energy electrons, also known as beta rays, that are emitted from an electron gun. The technology is analogous to an electron beam in a television tube, though far more powerful. Electrons can only penetrate several centimeters of food, and for this reason, foods are treated in relatively thin layers. Modest metal shielding of the treatment cell is sufficient to prevent the escape of stray electrons. When not in use, the electron source is turned off by switching off the electric current. No radioactivity is involved.

X-Irradiation

The most recently developed technology, X-irradiation, mixes properties of both of the above. High energy X-rays can be produced if an electron beam hits a thin metal foil target. Like gamma rays, a beam of X-rays can penetrate foods to a much greater

depth than electron beams and requires heavier shielding. However, like electron beams, X-ray sources can be switched on and off and do not use a radioactive source.

Effect of Irradiation on Microbes:

The high energy iradiation directly damage the DNA of living organisms, inducing cross-linkages and other changes that make an organism unable to grow or reproduce. When these rays interact with water molecules in an organism, they generate transient free radicals that can cause additional indirect damage to DNA. An absorbed dose of irradiation energy is now measured in Grays, rather rad. Complex life forms with large DNA molecules are affected by relatively low doses (31). Simpler organisms with smaller DNA can take progressively higher doses (Table 2.2). Thus, a low dose of under 0.1 kiloGray kills insects and parasites and inhibits plants from sprouting. A medium dose, between 1.5 and 4.5 kiloGray, kills most bacterial pathogens other than spores, and a higher dose of 10-45 kiloGray will inactivate bacterial spores and some viruses. For humans, the lethal dose is 4 Gray.

Table 2.2 Doses required to decrease selected pathogens at refrigerator temperatures by one decimal log/90% (D-dose)

Pathogens	D-dose in kGray*	5-log reduction dose in kGrays
Campylobacter	0.20	1.00
Toxoplasma cysts	0.25	1.25
E. coli O157	0.30	1.50
Listeria	0.45	2.25
Salmonella	0.70	3.50
Cl. botulinum spores	3.60	18.00

*1 Gray = 100 rads; 10 kGray = 1 Megarad

Irradiation has been approved for use on a broad range of foods for different purposes (Table 2.3). Irradiation on food was formally approved as though it were something added to food, rather than a process to which the food is subjected. This means that for meats and poultry, approval is required from both the FDA and USDA. The effect of irradiation on food itself is usually minimal at doses up to 7.5 kGy. Treated food does not become radioactive, and, in general, shelf life is prolonged because organisms that cause spoilage are reduced along with pathogens. Irradiation has been used effectively in meats, poultry, grains, and produce. However, not all foods can be irradiated without changing their quality. Meats with a high fat content may develop off-odors; the whites of eggs may go milky and liquid; and grapefruit gets mushy.

Table 2.3 Irradiation approved for foods in the United States

Year	Food	Dose (kGy)	Purpose
1963	Wheat flour	0.20-0.50	Control mold
1964	White potatoes	0.05-0.15	Inhibit sprouting
1986	Pork	0.30-1.00	Reduce cases of Trichinosis
1986	Fruits & vegetables	1.00	Increase shelf life and control insects
1986	Herbs and spices	30.00	Sterilize
1990 (FDA)	Poultry	3.00	Reduce bacterial pathogens
1992 (USDA)	Poultry	1.50-4.50	Reduce bacterial pathogens
1997 (FDA)	Fresh meat	4.50	Reduce bacterial pathogens
2000 (USDA)	Fresh meat	4.50	Reduce bacterial pathogens

Food Irradiation, Safety vs. Profits:

Irradiation is an ionized radiation process in order to kill bacteria. It is a safe process but it actually produces carcinogenic chemicals in certain foods. Studies of laboratory animals fed irradiated food developed chromosomal damage, tumors, kidney damage, and other problems.

Decades of research by reputable scientists indicate that irradiation will not lead to chemical changes in food that would adversely affect human health, will not lead to changes in the micro flora of food that would increase the microbiological risk, and will not lead to nutrient losses that would adversely affect the nutritional status of individuals or populations. Irradiation helps to ensure a safer and more plentiful food supply by extending shelf life and controlling pests and pathogens in foods. The absorbed dose the amount of energy absorbed by a food) is measured in units called kilo Grays (kGy). Low doses, less than one kGy, inhibit the sprouting of tubers, delay the ripening of some fruits and vegetables, control insects in fruits and stored grains, and reduce the problems of parasites in products of animal origin. Medium doses, 1-10 kGy, control pathogenic microbes responsible for food borne illness and extend the shelf life of refrigerated foods, thus allowing consumers to store foods longer and have a greater variety of fresh foods available in a larger market. High doses, greater than 10 kGy, are not yet being commercially used for foods other than spices and dried vegetable seasonings. Once higher doses are commercially accepted, consumers will have shelf stable foods that are of better quality than heat-sterilized, shelf stable foods.

Irradiation on Spices and herbs

Spices and herbs are values for their distinctive flavors, colors and aromas. Unfortunately, They are often contaminated with levels of bacteria, molds and yeasts; if untreated, they will result in rapid spoilage of foods.

In 1983, approval was granted to kill insects and control microorganisms in a specific list of herbs, spices and vegetable seasonings. In the US over 6.5 million of spices, herbs and dry ingredients are irradiated each year (30). The irradiation of spices and herbs is allowed in most countries. International standards have accepted it is a beneficial treatment.

CHAPTER 3
MATERIALS AND METHODS

1. Accessory for sample preparation

(1) Solvent Extraction Glassware
- Soxhlet 250 ml. and condenser
- Round Bottom Flask 1000 ml. Pyrex

(2) Heating Mantle

(3) Cooling system

(4) Vacuum Evaporator

(5) Evaporating Flask

(6) Suction Flask and Pump

2. Accessory for Chromatographic station

(1) High Performance Liquid Chromatography Column

Specification : Prevail C18 5μ 250 mm x 4.6 mm Alltech

type : Reverse phase C18 column

Lot No. : 2866

SN : 01120186.1

(2) Peristaltic Pump and Delivery System

Model : Waters 510, Waters 501

(3) Detector (Ultra-Violet Absorption Type)

Model : Waters 490E

(4) Gradient Controller

Model : Waters 680

(5) Computer Processor

3. Equipment

Cobalt-60 gamma ray source with an emission rate of 1.56 kGy/h

4. Chemicals and Solvents

(1) Hexane (Commercial grade)
(2) Chloroform (AR grade) Apex Chemical
(3) Acetronitrile (HPLC grade) J.T.Baker
(4) Methanol (HPLC grade) Labscan Co.Ltd.
(5) Phosphoric acid (AR grade) J.T.Baker
(6) Ethyl Acetate (AR grade) Labscan Co.Ltd.
(7) Ethanol (HPLC grade) Merck Co.Ltd.
(8) Standard andrographolide (purity 99.90%) was obtained from Chemical Synthesis Section; Department of Medical Science.

5. Methods

5.1 Plant materials

Dried specimens of *A. paniculata* leaf grounded into powder were purchased from 3 different local markets in Bangkok. Each specimen (A, B, C) was divided into control and study groups. Tripicates from each study group were irradiated at 5, 10 and 25 kGy by exposing to Co-60 gamma ray.

5.2 Extracted Preparation

(1) Weigh 15.0 g of dried sample powder into the filter bag and put in the soxhlet.
(2) Sequentially extract with hexane for 24 hours and then with chloroform for 72 hours.
(3) Filter the sample through the 0.45 μm membrane.
(4) Evaporate the solvent to adjust volume to 200 ml.
(5) Aliquots 1.0 ml of extracts were dried by rotary evaporator and store in refrigerator in a dry stage until use.
(6) Dry extracts were dissolved in 2 ml of ethyl-acetate and ethanol mixture (20:80) and adjust volume to 10.0 ml in a volumetric flask with deionized water.

5.3 Identification of extracts by Thin Layer Chromatography

The constituents of extracts are identified by simultaneously running standards with the unknown. One edge of the plate is then placed in a solvent reservoir and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The separated spots are visualized with ultraviolet light.

5.4 Measurement of andrographolide in Andrographis paniculata extracts by HPLC

Chromatographic conditions are as follows:

Mobile Phase solvents : Acetronitrile, 0.1% Phosphoric acid in water

Wavelength : UV 228 nm.

Flow Rate : 1.0 ml./min

Injection Volume : 20.0 μl

Run Time : 20 minutes

Mobile Phase : gradient programmed

Time (minutes)	% water with phosphoric acid	% Acetronitrile
0	70	30
2	65	35
6	60	40
12	23	77
17	40	60
20	70	30

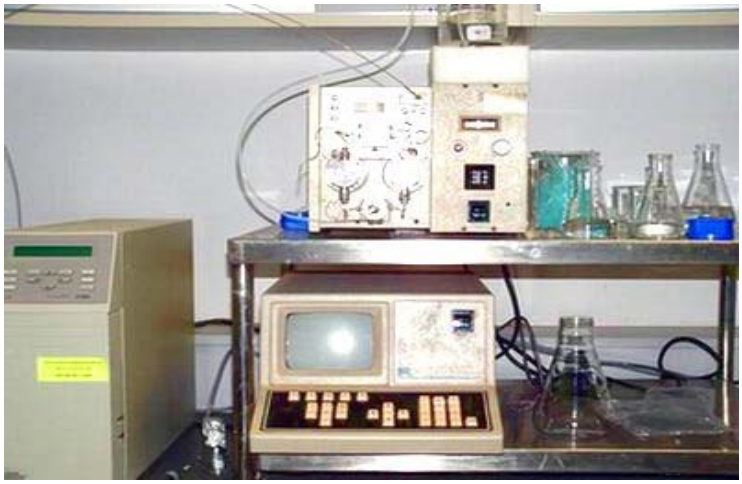


Figure 3.1 The HPLC Instrument includes pump, injector, gradient controller and detector.

Figure 3.2 The HPLC column; Alltech C18 RPC, dimension 250 mm. x 4.6 mm.

5.5 Standard Curve

Create the standard calibration curve for determining the concentration of sample by extrapolation on X-axis as shown in Figure 3.3.

Five concentrations of standard andrographolide, 0.031, 0.0625, 0.125, 0.200, 0.250 mg/ml were injected into HPLC system. The peak area values were obtained and plotted as a function of the standard concentrations; X axis is the standard concentration (milligram per milliliter; ppm), Y axis is a peak area unit.

Draw a line intercept on X axis and calculate the slop (a) from the linear equation; **Y = aX**

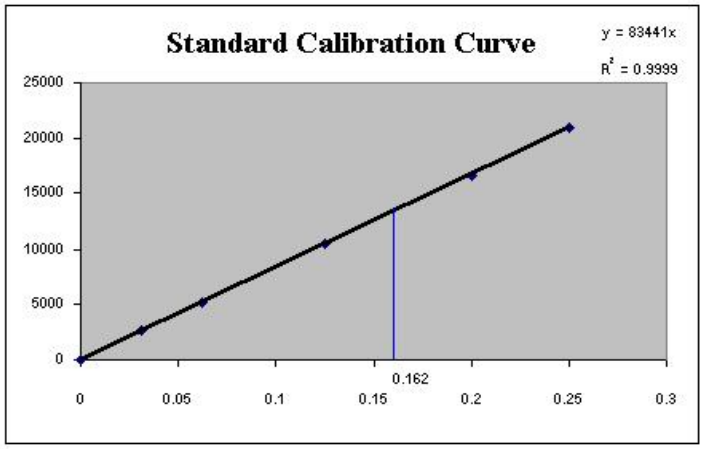


Figure 3.3 The standard calibration curve

5.6 Calculation

5.6.1 To determine slope of the straight line calibration curve:

$$\text{slope} = \frac{\Delta Y}{\Delta X}$$

5.6.2 The linear equation of the calibration curve is:

$$Y = aX + b \qquad (b=0)$$

$$\therefore \quad X = Y/a \qquad (a=\text{slope})$$

5.6.3 To determine sample concentration

if X = a mg/ml

Concentration of sample is = a mg.

20 µl derive from 10 ml volume concentration that it is the same concentration.

concentration of 10 ml volume derive from concentration of 1 ml volume

Hence 1 ml from initial concentration; 200 ml have AP = c mg.

Initial concentration; 200 ml have AP = c x 200 = d mg.

initial concentration ; 200 ml have AP d mg derive from sample about 15 g.

Sample powder 15 grams have AP = $d \times 10^{-3}$ g.

$$\text{Percentage of AP in sample} = \frac{d \times 10^{-3} \times 100}{15}$$

$$\therefore \quad \text{yield} = Z\ \%$$

The overall sample preparation process are shown in Figure 3.4

5.7 Sample analyses

Irradiated sample and controlled sample are quantitate for the content of andrographolide by the above condition. The percentage of andrographolide in irradiated sample are compared with the controlled. Statistical analysis of the difference between groups was done by t-test.

Sample preparation procedure chart

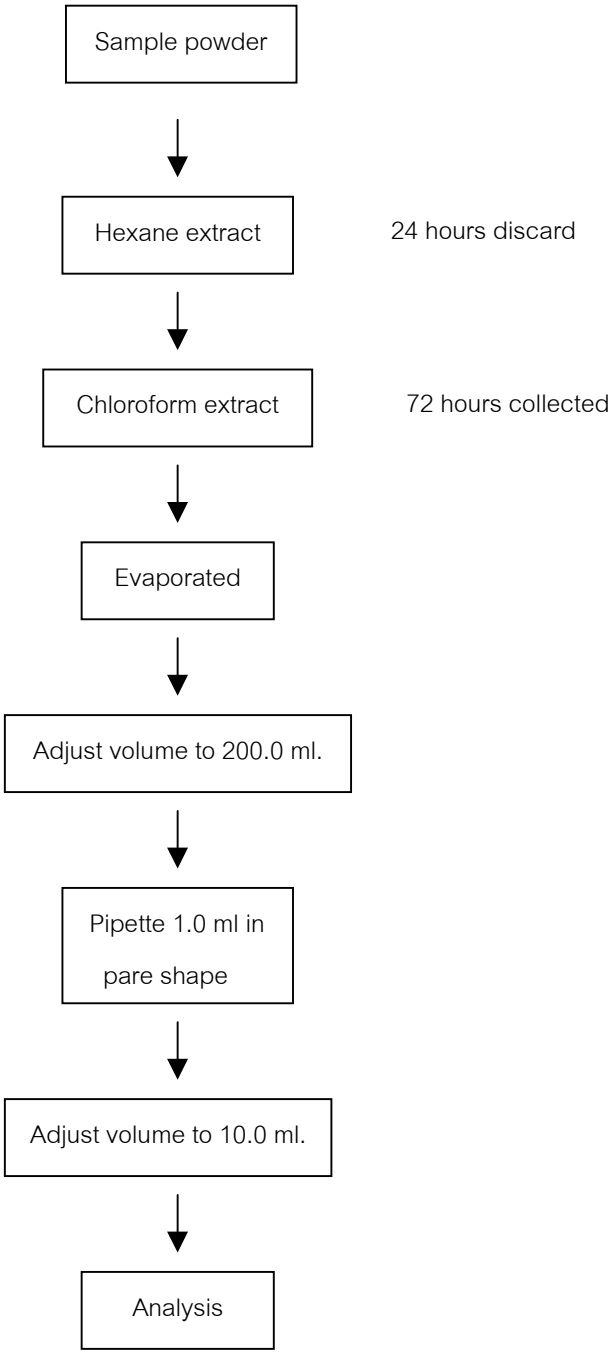


Figure 3.4 The process of sample preparation diagram

CHAPTER 4
RESULTS

4.1 Identification of the extracts by Thin layer chromatography (TLC)

Andrographolide extract was preliminary identified by carrying out TLC in 2 different solvent system and chromatography along with the reference standard andrographolide as shown in Figure 4.1.

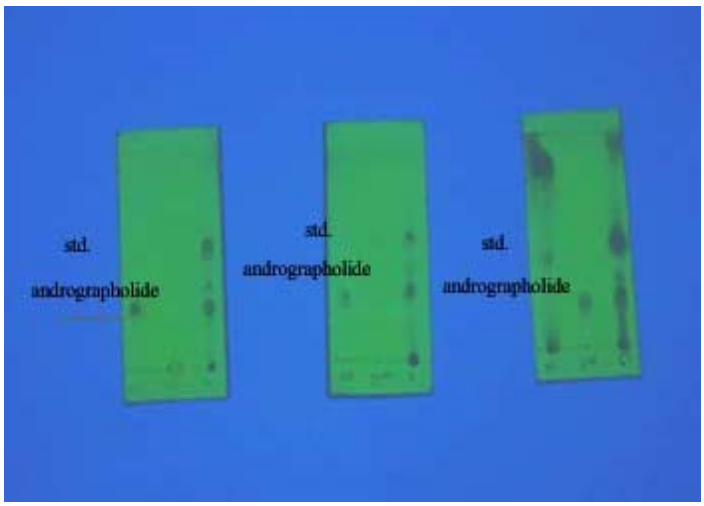


Figure 4.1 TLC of andrographolide extract and a reference standard held under UV lamp

4.2 Identification and quantitation of the andrographolide extracts by HPLC

Andrographolide was further indentified by carrying out HPLC of the extracts. The chromatogram of sample A, B and C and a reference standard by UV detection at a fix wavelength of 228 nm and a 20 microlitre of injected volume were shown in Figure 4.2, 4.3, 4.4 and 4.5 respectively.

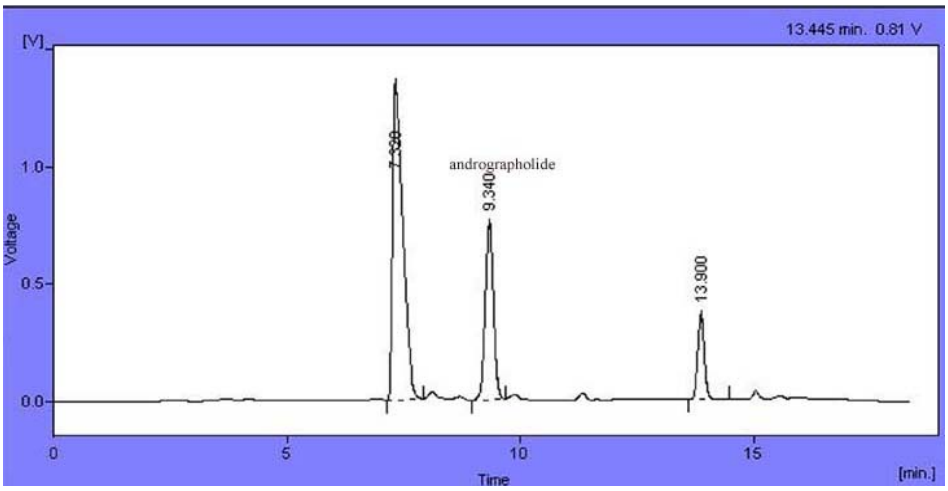


Figure 4.2 Illustrate the chromatogram of sample A by UV detection at 228 nm., Injection volume 20 µl.

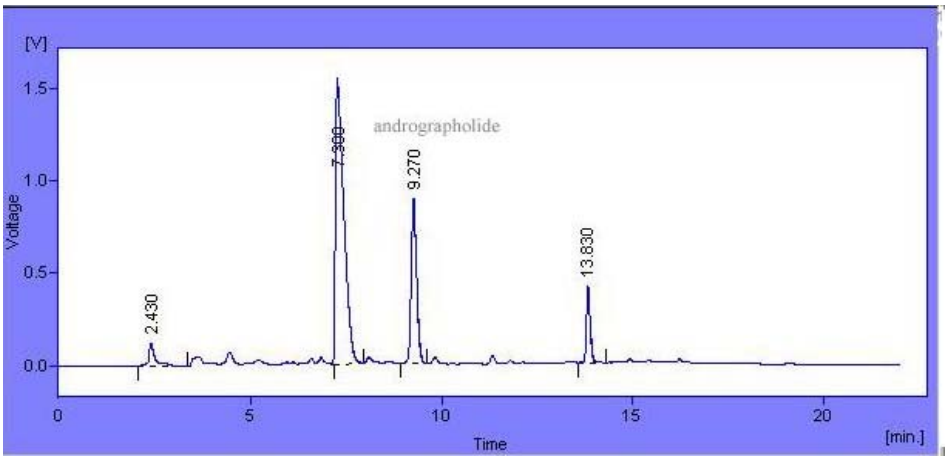


Figure 4.3 Illustrate the chromatogram of sample B by UV detection at 228 nm., Injection volume 20 µl.

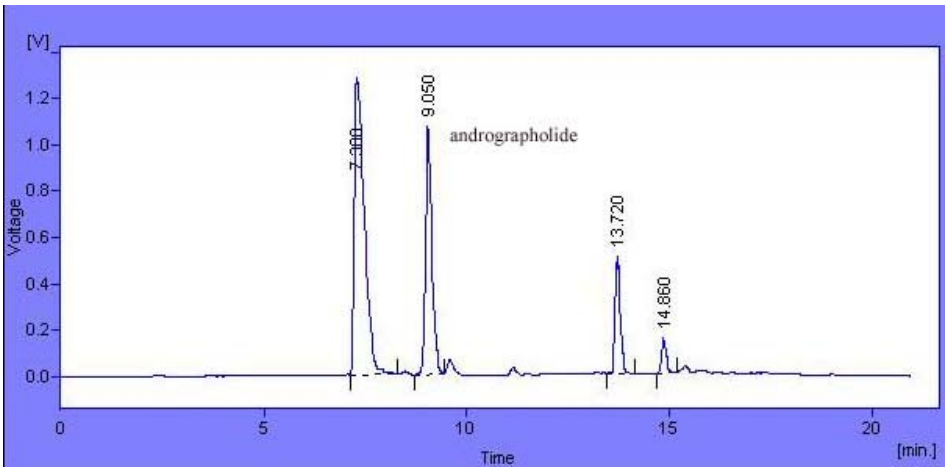


Figure 4.4 Illustrate the chromatogram of sample C by UV detection at 228 nm., Injection volume 20 µl.

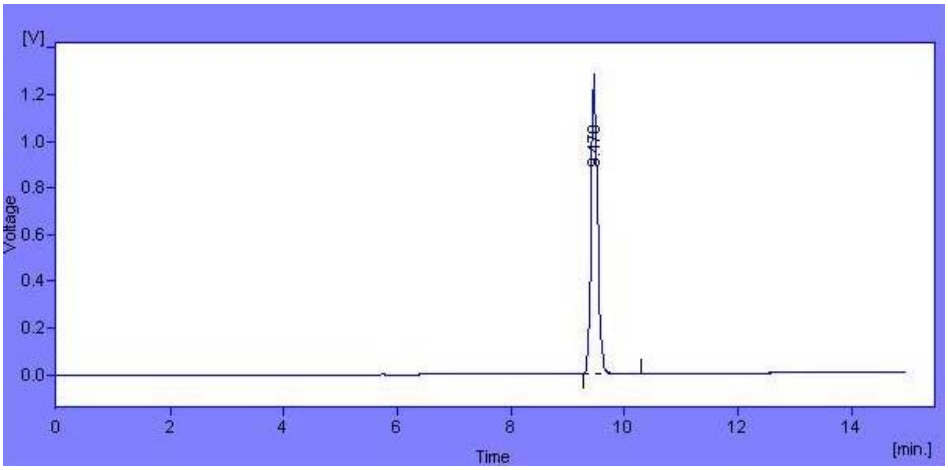


Figure 4.5 Illustrate the chromatogram of standard andrographolide by UV detection at 228 nm., Injection volume 20 µl.

The data obtained from chromatography were processed by auto-calculation program software. Raw data of HPLC peak area of the irradiated and controlled samples A, B and C were shown in Table B2, B3 and B4 in appendix B.

The concentration of andrographolide in mg/ml. Was calculated from the standard calibration curve. The linear relationship between peak area and

andrographolide concentrations was shown by the equation " Y = aX" ,where Y is the peak area unit and X is the concentration in mg/ml. The raw data of the concentration were tabulated in Table B5, B6, B7 in appendix B.

The yield of andrographolide extract in percentage (ratio between the dried starting material and the final recorded concentration) ranged from 1.3875 to 1.4437, 1.5252 to 1.5768, 2.0740 to 2.2609 for sample A, B and C respectively .

Comparisons with control groups at difference dose levels and storage time were summarized in Table 4.1.

Table 4.1 The summarized results of percentage of andrographolide contents of sample A, B and C over a 50 day storage time

SAMPLE A						
	Dose at 5 kGy		Dose at 10 kGy		Dose at 25 kGy	
	controlled	irradiated	controlled	irradiated	controlled	irradiated
Average	1.4372	1.4269	1.4378	1.4310	1.4168	1.4074
SD	0.0099	0.0123	0.0105	0.0091	0.0173	0.0193
SAMPLE B						
	Dose at 5 kGy		Dose at 10 kGy		Dose at 25 kGy	
	controlled	irradiated	controlled	irradiated	controlled	irradiated
Average	1.5549	1.5550	1.5549	1.5500	1.5623	1.5518
SD	0.0185	0.0205	0.0185	0.0192	0.0143	0.0131
SAMPLE C						
	Dose at 5 kGy		Dose at 10 kGy		Dose at 25 kGy	
	controlled	irradiated	controlled	irradiated	controlled	irradiated
Average	2.2301	2.2214	2.1595	2.1549	2.1392	2.1329
SD	0.0375	0.0346	0.0493	0.0458	0.0520	0.0548

4.3 Effect of storage time on andrographolide concentrations

Samples A, B and C irradiated with gamma ray dose of 5, 10 and 25 kGy and the controls were sequentially analyzed at 4, 7, 14, 28 and 50-day storage periods.

Average concentrations of andrographolide ± SD of each individual sample irradiated at different dose levels and different storage periods from 4 up to 50 days was tabulated with the controls as shown in Table 4.1 and Figure 4.6 to 4.14

There were no differences statistically between samples and control groups either at different dose levels or different storage period shown in Table 4.2.

Table 4.2 The t-test results of sample A, B and C

SAMPLE A			
t-calculated	Dose at 5 kGy	Dose at 10 kGy	Dose at 25 kGy
At 4 days	0.9366	0.7653	1.3939
At 7 days	2.0547	2.1591	0.8730
At 14 days	2.0596	1.3605	0.9241
At 28 days	0.0285	0.0047	0.0245
At 50 days	2.0225	0.8664	0.3600
SAMPLE B			
t-calculated	Dose at 5 kGy	Dose at 10 kGy	Dose at 25 kGy
At 4 days	-0.1391	0.1711	0.6536
At 7 days	-0.1822	0.1424	0.4290
At 14 days	-0.0856	0.2307	1.7062
At 28 days	0.2632	0.5258	1.9130
At 50 days	0.2417	0.2502	0.2127
SAMPLE C			
t-calculated	Dose at 5 kGy	Dose at 10 kGy	Dose at 25 kGy
At 4 days	1.0912	-0.0322	1.6490
At 7 days	1.5937	2.0068	2.1320
At 14 days	1.8373	1.0594	-0.7211
At 28 days	1.5450	0.1606	2.1939
At 50 days	1.9858	1.7272	1.8646

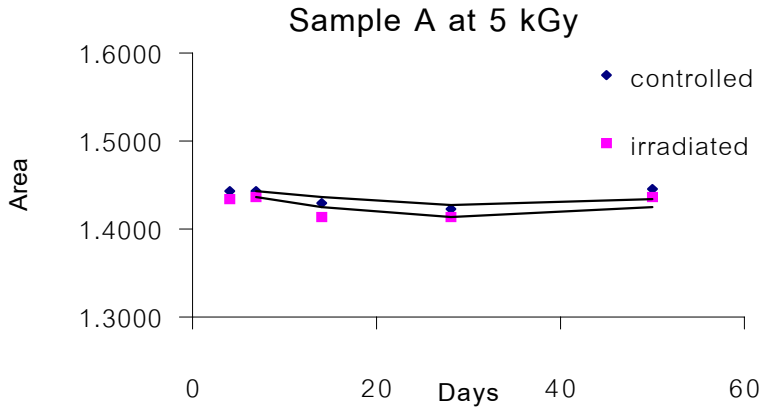


Figure 4.6 Andrographolide content as a function of time up to 50 days after irradiation of Sample A at 5 kGy

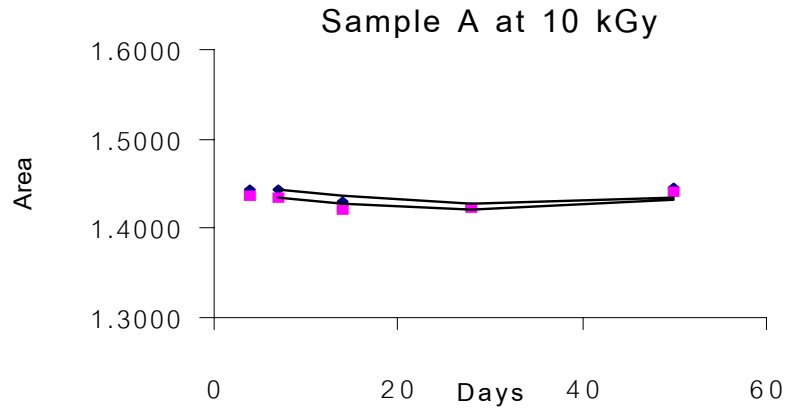


Figure 4.7 Andrographolide content as a function of time up to 50 days after irradiation of Sample A at 10 kGy

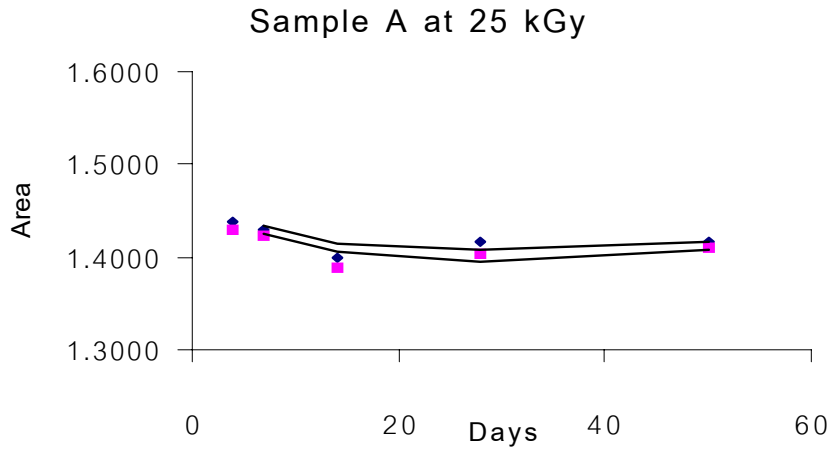


Figure 4.8 Andrographolide content as a function of time up to 50 days after irradiation of Sample A at 25 kGy

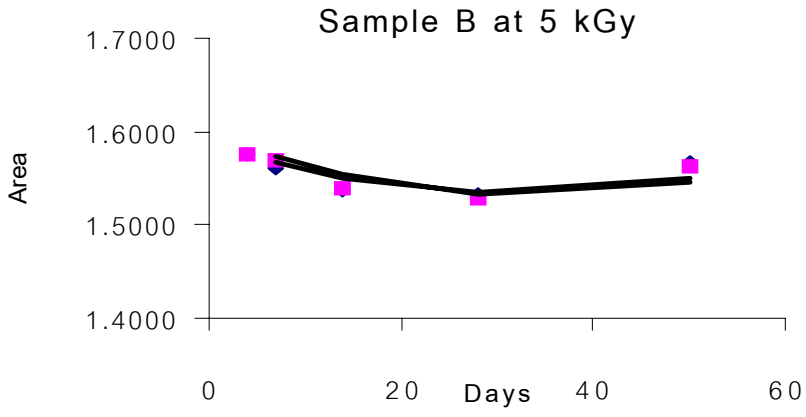


Figure 4.9 Andrographolide content as a function of time up to 50 days after irradiation of Sample B at 5 kGy

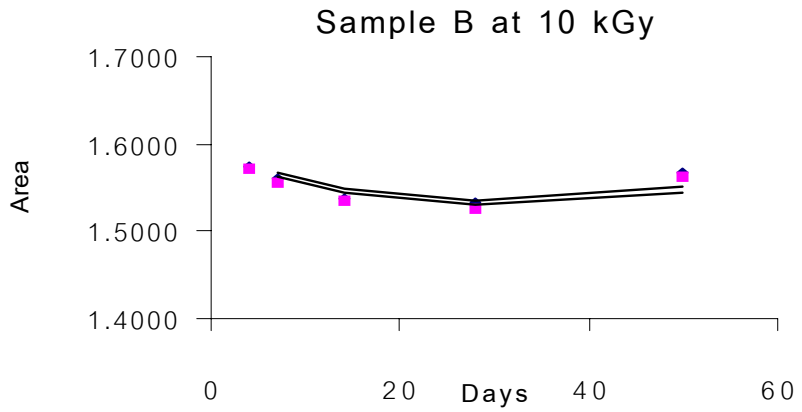


Figure 4.10 Andrographolide content as a function of time up to 50 days after irradiation of Sample B at 10 kGy

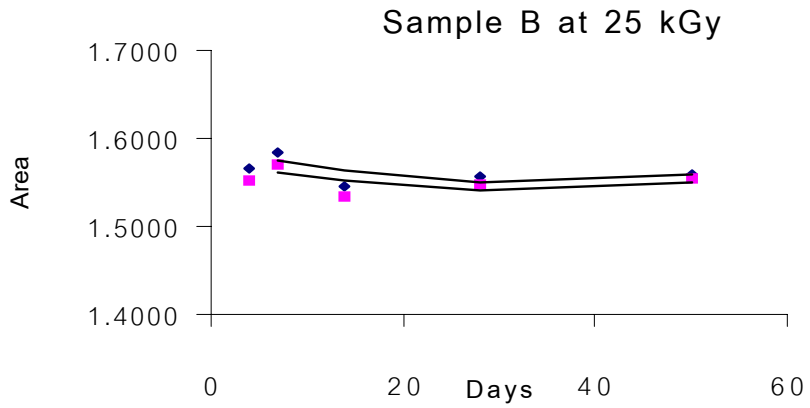


Figure. 4.11 Andrographolide content as a function of time up to 50 days after irradiation of Sample B at 25 kGy

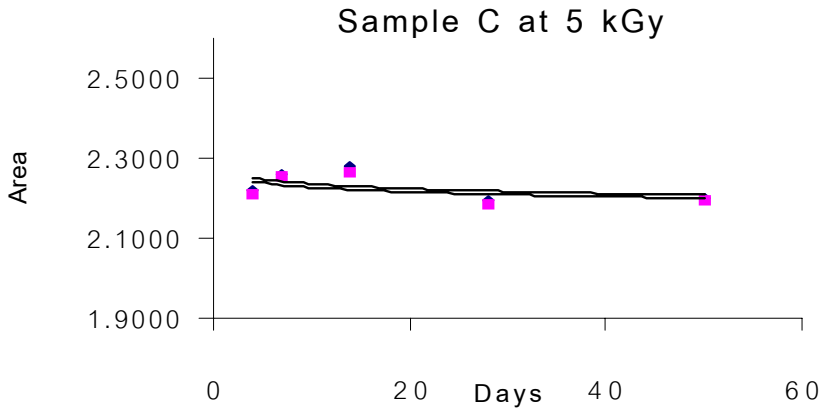


Figure 4.12 Andrographolide content as a function of time up to 50 days after irradiation of Sample C at 5 kGy

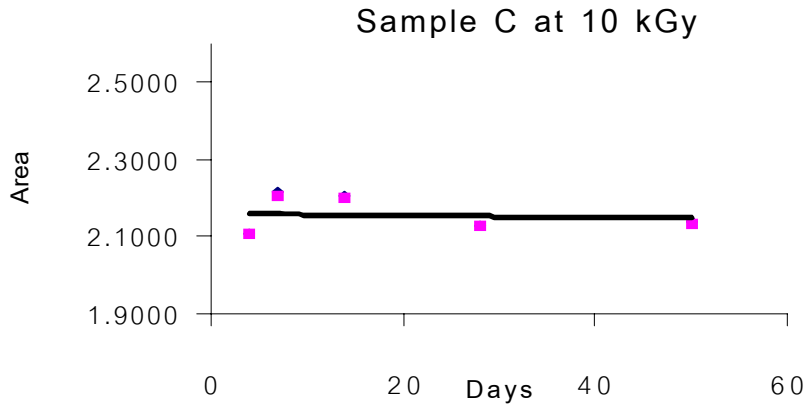


Figure 4.13 Andrographolide content as a function of time up to 50 days after irradiation of Sample C at 10 kGy

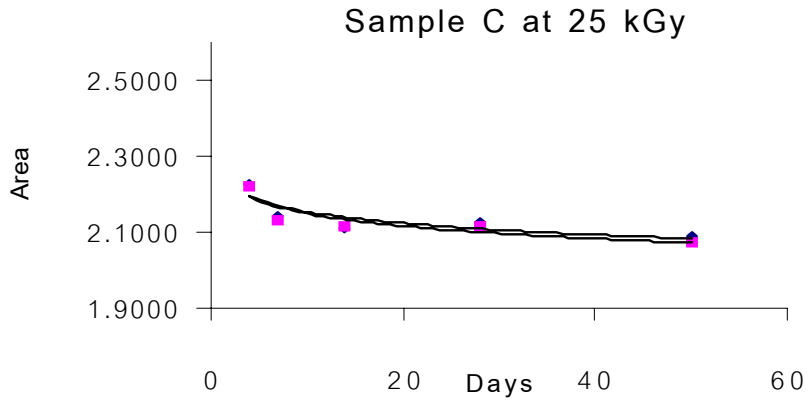


Figure 4.14 Andrographolide content as a function of time up to 50 days after irradiation of Sample C at 25 kGy

4.4 Dose dependent effect on andrographolide concentrations

Andrographolide in the extracts determined after samples A, B and C were irradiated with Co-60 gamma ray dose of 5, 10 and 25 kGy were found to be comparable in the amounts with the controlled sample. A statistical analysis performed using unpaired two-tailed t-test do not show any significant differences (Figure 4.15, 4.16, 4.17).

Table 4.3 A comparison between the percentage of andrographolide content of irradiated samples A, B and C and the controlled (dose compared). (see full data at appendix II)

Sample	A		B		C	
	controlled	irradiated	controlled	irradiated	controlled	irradiated
Average	1.4315	1.4228	1.5574	1.5523	2.1763	2.1697
SD	0.0137	0.0150	0.0164	0.0167	0.0591	0.0576
t-test	1.6608		0.8416		0.3061	

* * t-test table (2-way) : $t_{0.025} = 2.145$, $t_{-0.025} = -2.145$

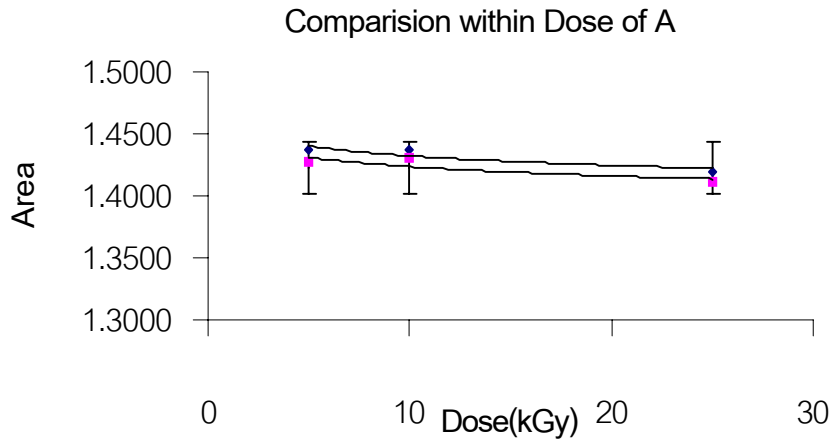


Figure. 4.15 Andrographolide content after irradiation of sample A as a function of radiation dose from 5 to 25 kGy

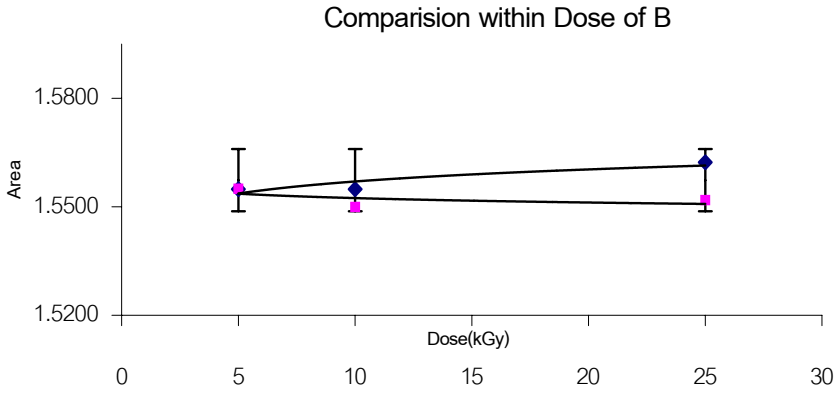


Figure. 4.16 Andrographolide content after irradiation of sample B as a function of radiation dose from 5 to 25 kGy

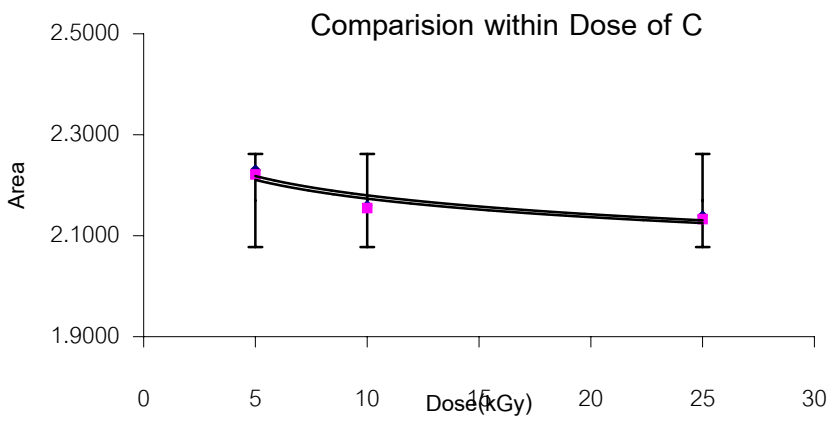


Figure. 4.17 Andrographolide content after irradiation of sample C as a function of radiation dose from 5 to 25 kGy

4.5 Precision of measurements

The precision of andrographolide measurement was studied by determining the intra- and inter-batches varieties. Average of the percentage concentration of andrographolide ± SD and percentage coefficient of variation (% CV) in the same and different chromatograms were tabulated in Table 4.4.

The % CV in the same and different chromatograms were 0.64 and 2.56.

Table 4.4 Intra and inter batches varieties.

	%Andrographolide	
	Intra-batch	Inter-batch
Average	1.3751	1.5193
SD	0.0088	0.0389
%CV	0.64	2.56

CHAPTER 5
DISCUSSION AND CONCLUSION

In this paper an HPLC method was employed to quantitate the content of andrographolide, an active ingredient from crude extracts of *A. paniculata.* Our studies revealed that the HPLC demonstrated to be appropriate for quantitative analysis of the active component in *A. paniculata* with respect to separation efficiency and reproducibility. A good chromatographic separation was clearly exhibited within a 20 minute run time.

A HPLC procedure has been used to examine the effect of Co-60 irradiation for controlling microorganisms and molds on dried leaf powder of *A. paniculata.* Despite the known benefits of irradiation, the radiation absorbed dose should not cause any significant change in the content of active ingredient. Our studied revealed that the active component is not affected by irradiation dose between 5 to 25 kGy as well as a storage period up to 50 days after irradiation. As shown in the results, irradiated samples A, B and C exhibited slightly decreased in contents of andrographolide after irradiation when compared to the control samples, however, the changes were not significant statistically. It is likely that the variations are contributed from differences in experimental conditions, instrumental and human errors rather than from the effect of radiation.

Upon storage period of 50 days, no significant loss of andrographolide was found with the treatment dose of 5, 10 and 25 kGy. It is concluded that the chemical structure of andrographolide had a high degree of stability over the period of 50 days (32).

The irradiation of spices and herbs is allowed in most countries and international standards have accepted it as a beneficial treatment to extend it shelf life. It has been reported that a minimum dose of 5 kGy can effectively kill bacteria, molds and yeast and the sensory properties of most spices and herbs are well maintained between 7.5 to 15 kGy. Our studies have shown that irradiation dose as high as 25 kGy does not cause any significant loss of andrographolide. It is concluded that irradiation of *A. paniculata* up to 25 kGy is validated for extending its shelf life.

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APPENDIX

APPENDIX A

Glossary of Chromatographic Terms

Adjusted retention time (t'_R) A measure of the retention time adjusted for the void volume.
Adjusted retention volume (V'_R). Adjusts the retention volume for the dead volume.
Adsorbent. Packing used in adsorption chromatography as an adsorbents in HPLC.
Band broadening. The dilution of the chromatographic band as it moves down the column. The peak is injected as a slug, and, if not for the process of band broadening, each separated component would elute as a narrow slug of pure compound. The measure of band broadening is band width, t_w, or more correctly, the number of theoretical plates in the column. N.
Band width (t_w) The width of the chromatographic band during elution from the column. It is usually measured at the baseline by drawing tangents to the sides of the Gaussian curve representing the peak.
Bonded-phase chromatography (BPC). The most popular LC mode. A stationary phase chemically bonded to a support is used for the separation.
Capacity factor. A chromatographic parameter that measures the degree of retention . See k ' for calculation method.
Chromatogram. A plot of detector signal output versus time or elution volume during the chromatographic process.
Column chromatography. Any form of chromatography that uses a column or tube to hold the stationary phase. Open-column chromatography, HPLC, and open-tubular capillary chromatography are all examples .
Cross-linking. During the process of copolymerization of resins to form a three dimensional matrix, a difunctional monomer is added to form cross-linkages hetween adjacent polymer chains. The degree of cross-linking is determined by the amount of this monomer added to the reaction. For example, divinylbenzene is a typical cross-linking agent for polystyrene ion-exchange resins. The swelling and diffusion characteristics of a resin are governed by its degree of cross-linking.
Dead volume (V_d). The volume outside of the column packing itself. The interstitial volume (intraparticle volume + interparticle volume) plus extracolumnvolume (contributed by injector, detector, connecting tubing, and endfittings) all combine to create the dead volume. This volume can be determined by injecting an inen compound (i.e.. a compound that does not interact with the column packing). Also abbreviated V0 or Vm.
Diffusion coefficient *(D_m or D_s).* A fundamental parameter of a molecule in solution *(D_m)* or in stationary phase *(D_s).* Expressed in cm2/s. Dm depends on molecular weight of the solute, on temperature, on solvent viscosity, and on molar volume of the solute. A typical value of a small molecule in RPC at room temperature would be S x 10 cm^2/s.
Effective theoretical plates (Neff). The true number of plates in a column; corrects theoretical plates for dead volume. Neff = $16(t_R - t_m)^2$, where tm is the void time.

Efficiency *(N or H)*. A measure determined by the number of theoretical plates calculated from the equation shown for *H* (see HETP).
Fronting. Peak shape in which the front pan of a peak (before the apex) in a chromatogram tapers in advance of the remainder of the peak. There is an asymmetric distribution with a leading edge. The asymmetry factor for a fronting peak has a value < 1.
The opposite effect is tailing. Fronting is related to the shape of the sorption isotherm.
Gaussian curve. A standard error curve, based on a mathematical function. that is a symmetrical, bellshaped band or peak. Most chromatographic theory assumes a Gaussian peak.
Gradient elution. The two or more mobile phase strength over time during the chromatographic separation. Also known as solvent programming.
HETP. Height equivalent to a theoretical plate. A carryover from distillation theory: a measure of a column's efficiency. For a typical HPLC column well-packed with 5 µm particles, HETP (or *H*) values are usually between 0.01 and 0.03 mm.
Isocratic . Use of a constant-composition mobile phase in liquid chromatography.
Mass transfer. An event of solute movement into and out of the stationary phase or mobile phase. In HPLC, mass transfer is the most important factor affecting column efficiency. It is increased by the use of small-particle packings, thin layers of stationary phase, low viscosity mobile phases, and high temperatures.
Mobile phase. The solvent that moves the solute through the column.
Normal-phase chromatography. A mode of chromatography carried out with a polar stationary phase and a nonpolar mobile phase. Adsorption on silica gel using hexane as a mobile nhase would be a typical normal-phase system. Also ;efers to the use of polar bonded phases, such as CN or NH_2. Sometimes referred to as straight-phase chromatography.
Octadecylsilane (ODS = RP-18). The most popular reversed phase in HPLC. Octadecylsilane phases are bonded to silica or polymeric packings. Both monomeric and polymeric phases are available.
Partition chromatography. Separation process in which one of the liquid phases is held stationary on a solid support while the other is allowed to flow freely down the column. Solutes panition themselves between the two phases based on their individual partition coefficients. Liquid-liquid chromatography is an example.
Partition coefficient (K). The amount of solute in the stationary phase relative to the amount of solute in the mobile phase. Can be distribution coefficient, KD
Resolution (Rs). Ability of a column to separate chromatographic peaks. It is usually expressed in terms of the separation of two peaks. One attempts to achieve the best resolution possible.
Retention time (t_R). The time between injection andthe appearance of the peak maximum. The adjusted retention time t'_R adjusts for the column void volume. $t'_R = t_R - t_0$ (or t_m).
Retention volume (V_R) . The volume of mobile phase required to elute a substance from the column $V_R = F \bullet t_R$ or $V_R = V_m - K_D V_s$, where Vm is the void volume, KD the distribution coefficient. and Vs the stationary phase volume.
Reversed-phase chromatography (RPC). The most common HPLC mode. Uses hydrophobic packings such as octadecyl- or octylsilane phases bonded to silica or neutral polymeric beads. Mobile phase is usually water and a water-miscible organic solvent such as methanol or acetonitrile. There are many variations of RPC in which

various mobile phase additives are used to impan a different selectivity. For example, for the RPC of anions, the addition of a buffer and tetraalkylammonium salt would allow ion pairing to occur and effect separations that rival ion-exchange chromatography.

Stationary phase. The immobile phase involved in the chromatographic process. The stationary phase in liquid chromatography can be a solid, a bonded or coated phase on a solid Suppon, or a wall-coated phase. The stationary phase used ohen characterizes the LC mode. For example. silica gel is used in adsorption chromatography, an octadecylsilane bonded phase in reversed-phase chromatography, etc.

Tailing. The phenomenon in which the normal Gaussian peak has an asymmetry factor > 1. The peak wil I have skew in trailing edge. Tailing is caused by sites on the packing that have a stronger-than normal retention for the solute. A typical example of a tailing phenomenon is the strong adsorption of amines on the residual silanol groups of a low-coverage reversed-phase packing.

Void volume (V_i). The total volume of mobile phase in the column: the remainder of the column is taken up by packing material. Can be determined by injecting an unretained substance that measures void volume plus extracolumn volume. Also referred to as interstitial volume. V_0 or V_m are sometimes used as symbols.

APPENDIX B

1. Raw data of HPLC peak area for Standard Calibration Curve creation.
Table B1. Data of HPLC peak area of Standard Andrographolide for Standard Calibration Curve by date to date

Concentration	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2730.25	2639.098	2689.61	2719.499	2716.789	2745.98
0.063 mg/ml	5377.88	5349.613	5380.66	5335.285	5213.013	5459.38
0.125 mg/ml	10403.71	10261.75	10390.26	10112.87	10106.40	10342.7
0.200 mg/ml	16380.24	16478.86	16007.06	15851.41	15905.80	16129.3
0.250 mg/ml	20800.01	20361.17	19272.36	20436.08	20208.53	20436.7
Y=aX	82611	81997	79175	80990	80547	81747
R^2	0.9994	0.9997	0.9973	0.9991	0.9996	0.9993

Concentration	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2675.682	2704.292	2772.006	2739.030	2709.254	2778.47
0.063 mg/ml	5445.296	5382.904	5466.782	5427.261	5365.717	5570.69
0.125 mg/ml	10496.53	10173.08	10348.23	10469.17	10162.86	10403.0
0.200 mg/ml	16289.21	16066.17	16329.96	16258.42	16183.22	16713.2
0.250 mg/ml	20603.24	20485.79	19877.90	20497.08	20833.35	21378.1
Y=aX	82477	81522	80953	82190	82401	84736
R^2	0.9995	0.9994	0.9986	0.9994	0.9993	0.9993

Concentration	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2695.142	2768.854	2736.658	2745.178	2802.373	2831.43
0.063 mg/ml	5395.089	5010.652	5091.78	5118.85	5112.55	5148.81
0.125 mg/ml	10418.70	10231.27	10051.65	10091.59	10000.57	9853.71
0.200 mg/ml	15690.58	16563.26	16332.03	16540.93	16303.21	16143.5
0.250 mg/ml	20236.20	20454.87	19923.87	20192.26	19763.52	20175.24
Y=aX	80658	82154	80549	81491	80151	80605
R^2	0.9984	0.9997	0.9995	0.9995	0.9991	0.9994

Concentration	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2728.4895	2884.399	2622.882	2584.562	2612.693	2612.693
0.063 mg/ml	5133.154	5238.484	5201.4095	5181.974	5136.381	5136.381
0.125 mg/ml	9722.5595	10081.28	10072.611	10195.34	10283.99	10283.99
0.200 mg/ml	15382.552	16296.75	15990.121	16667.32	16606.96	16606.96
0.250 mg/ml	19649.935	20363.18	20546.716	20290.90	20685.02	20685.02
Y=aX	78133	81526	81307	81994	82771	82771
R^2	0.9992	0.9995	0.9995	0.9996	1	1

Concentration	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2589.3345	2589.334	2614.9435	2631.479	3073.205	3063.9935
0.063 mg/ml	5247.359	5247.359	5168.778	5203.306	5201.424	5287.44
0.125 mg/ml	10302.507	10302.10	10487.864	10560.016	10498.076	10517.062
0.200 mg/ml	16786.476	16786.47	16775.934	16526.391	16738.086	17196.773
0.250 mg/ml	20581.104	20581.10	20210.45	20921.912	20460.692	20555.795
Y=aX	82921	82921	82305	83441	82895	83895
R^2	0.9998	0.9998	0.9991	0.9999	0.9989	0.9983

Concentration	Peak area	Peak area	Peak area	Peak area
0.032 mg/ml	2610.682	2396.2955	2494.679	2824.395
0.063 mg/ml	5152.816	5025.6805	4786.464	5052.423
0.125 mg/ml	10304.463	10243.474	9737.323	10195.185
0.200 mg/ml	16504.137	16004.95	15890.22	16443.644
0.250 mg/ml	20431.084	20165.288	19803.20	20492.73
Y=aX	82116	80583	79050	82035
R^2	0.9999	0.9998	0.9998	0.9997

2. Data of HPLC peak area of irradiated sample and controlled sample

All of this data obtained from chromatography process by auto-calculation with specified software

Table B2. Raw data of HPLC peak area of irradiated sample and controlled sample A

Sample A	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	8804.689	8775.100	8804.689	8753.756	8518.144	8452.833
	4	8781.725	8778.575	8781.725	8761.164	8513.669	8405.494
	4	8814.579	8676.878	8814.579	8744.39	8518.988	8490.039
set 2	4	8796.181	8722.002	8796.181	8717.64	8796.181	8746.625
	4	8660.211	8694.14	8660.211	8732.532	8660.211	8726.571
	4	8761.829	8646.957	8761.829	8647.801	8761.829	8636.73
set1	7	8941.385	8905.27	8941.385	8923.108	8539.503	8508.575
	7	8913.575	8916.878	8913.575	8884.119	8525.635	8486.818
	7	8918.447	8887.946	8918.447	8815.256	8533.163	8500.132
set 2	7	8811.765	8791.156	8811.765	8770.935	8811.765	8774.55
	7	8947.896	8842.175	8947.896	8878.091	8947.896	8812.754
	7	8895.432	8822.445	8895.432	8803.454	8895.432	8876.422
set1	14	8885.509	8836.446	8885.509	8806.164	8502.272	8435.186
	14	8878.645	8818.768	8878.645	8842.825	8458.464	8410.541

	14	8909.027	8827.482	8909.027	8885.038	8478.754	8373.96
set 2	14	8843.919	8723.452	8843.919	8717.916	8843.919	8802.355
	14	8829.548	8690.128	8829.548	8809.042	8829.548	8713
	14	8836.132	8704.638	8836.132	8773.454	8836.132	8755.55
set1	28	8716.322	8581.284	8716.322	8732.32	8652.527	8611.688
	28	8681.823	8668.862	8781.823	8758.621	8627.781	8533.08
	28	8732.767	8693.209	8832.767	8771.294	8642.048	8571.131
set 2	28	8859.80	8841.306	8859.80	8839.113	8859.80	8861.858
	28	8899.57	8896.111	8899.57	8906.114	8899.57	8781.089
	28	8993.792	8800.512	8993.792	8829.256	8993.792	8829.65
set1	50	8994.108	8901.708	8994.108	8915.657	8387.369	8405.873
	50	8955.286	8909.145	8955.286	8977.659	8453.871	8394.455
	50	8938.066	8891.193	8938.066	8971.294	8407.425	8393.498
set 2	50	8827.028	8743.538	8827.028	8787.165	8827.028	8881.089
	50	8855.156	8842.342	8855.156	8830.877	8855.156	8746.625
	50	8891.407	8832.194	8891.407	8801.213	8891.407	8805.55

Table B3. Raw data of HPLC peak area of irradiated sample and controlled sample B

Sample B	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	9330.972	9355.347	9330.972	9352.404	9498.015	9317.145
	4	9344.572	9339.420	9344.572	9382.084	9396.924	9357.071
	4	9368.933	9411.045	9368.933	9215.272	9410.701	9313.558
set 2	4	9624.588	9637.457	9624.588	9613.949	9624.588	9610.598
	4	9672.447	9655.237	9672.447	9682.603	9672.447	9571.721
	4	9686.032	9702.471	9686.032	9681.045	9686.032	9604.913
set1	7	9325.570	9297.793	9325.57	9267.693	9396.497	9321.199
	7	9271.976	9342.88	9271.976	9213.424	9390.399	9278.259
	7	9232.341	9360.30	9232.341	9303.861	9321.544	9304.844
set 2	7	9711.636	9691.349	9711.636	9602.827	9711.636	9617.009
	7	9666.105	9682.331	9666.105	9590.144	9666.105	9540.806
	7	9644.799	9769.355	9644.799	9654.676	9644.799	9610.273
set1	14	9263.75	9283.575	9263.75	9248.377	9385.05	9345.403
	14	9249.671	9318.366	9249.671	9270.477	9494.991	9381.85
	14	9255.431	9224.386	9255.431	9217.118	9396.574	9355.149
set 2	14	9568.863	9609.254	9568.863	9657.535	9568.863	9618.212
	14	9664.748	9651.318	9664.748	9553.047	9664.748	9552.586
	14	9659.157	9600.937	9659.157	9636.181	9659.157	9537.219
set1	28	9305.377	9259.477	9305.377	9272.989	9470.471	9363.488
	28	9299.026	9236.815	9299.026	9272.798	9423.144	9270.415
	28	9274.165	9317.497	9274.165	9273.266	9352.974	9355.288
set 2	28	9720.879	9752.757	9720.879	9684.849	9720.879	9689.091
	28	9723.652	9689.355	9723.652	9648.101	9723.652	9672.77
	28	9725.253	9633.873	9725.253	9604.94	9725.253	9740.806

set1	50	9243.767	9276.078	9243.767	9354.791	9347.799	9392.075
	50	9360.096	9329.000	9360.096	9369.394	9432.269	9314.919
	50	9372.42	9310.885	9372.42	9270.827	9420.506	9287.69
set 2	50	9737.877	9714.479	9737.877	9602.93	9737.877	9755.833
	50	9735.998	9602.017	9735.998	9600.065	9735.998	9645.639
	50	9643.687	9650.937	9643.687	9689.283	9643.687	9678.953

Table B4. Raw data of HPLC peak area of irradiated sample and controlled sample of sample C

Sample C	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	13673.12	13458.38	13225.37	13315.26	13513.87	13483.45
	4	13624.25	13652.06	13216.17	13176.98	13532.69	13494.16
	4	13708.66	13717.06	13314.69	13310.48	13498.02	13515.50
set 2	4	13673.12	13622.69	13225.37	13225.59	13513.87	13531.74
	4	13624.25	13694.40	13216.17	13256.82	13532.69	13472.40
	4	13708.65	13601.44	13314.69	13233.00	13498.02	13480.07
set1	7	13623.19	13518.21	13362.34	13328.92	13189.69	13184.84
	7	13642.03	13643.17	13379.19	13357.09	13255.64	13151.77
	7	13710.22	13699.91	13471.55	13389.46	13247.87	13232.00
set 2	7	13623.19	13577.66	13362.34	13359.41	13189.69	13073.98
	7	13642.03	13626.49	13379.19	13377.32	13255.64	13215.90
	7	13710.22	13595.87	13471.55	13328.05	13247.87	13188.37
set1	14	13706.73	13816.10	13416.09	13510.87	13127.96	13090.09
	14	13820.75	13734.34	13457.71	13496.75	12919.15	12991.67
	14	13787.81	13775.78	13597.22	13424.02	13012.69	13073.02
set 2	14	13706.73	13605.57	13416.09	13449.84	13127.96	13013.46
	14	13820.75	13608.20	13457.71	13464.08	12919.15	13101.83
	14	13787.81	13530.54	13597.22	13303.13	13012.69	13032.45
set1	28	13655.23	13687.62	13185.78	13267.13	13024.23	12938.39
	28	13684.91	13578.67	13178.60	13117.90	12963.47	12896.43
	28	13610.66	13611.09	13274.13	13260.94	12903.43	12911.95
set 2	28	13655.23	13650.59	13185.78	13194.12	13024.23	12906.77
	28	13684.91	13475.07	13178.60	13175.71	12963.47	12905.62
	28	13610.66	13595.70	13274.13	13232.03	12903.43	12922.10
set1	50	13591.83	13538.18	13184.71	13213.33	12846.01	12905.73
	50	13564.12	13592.60	13179.15	13160.96	12998.76	12819.51
	50	13544.50	13524.61	13196.90	13193.46	13096.86	12848.63
set 2	50	13591.83	13541.19	13184.71	13153.26	12846.01	12975.74
	50	13564.12	13503.76	13179.15	13088.37	12998.76	12819.27
	50	13544.50	13519.36	13196.90	13078.14	13096.86	12922.80

Table B5. The concentration of andrographolide (mg/ml) in sample A

Sample A	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	0.1093	0.1089	0.1093	0.1087	0.1085	0.1076
	4	0.1090	0.1090	0.1090	0.1088	0.1084	0.1070
	4	0.1094	0.1077	0.1094	0.1086	0.1085	0.1081
set 2	4	0.1079	0.1070	0.1079	0.1069	0.1079	0.1073
	4	0.1062	0.1066	0.1062	0.1071	0.1062	0.1070
	4	0.1075	0.1061	0.1075	0.1061	0.1075	0.1059
set1	7	0.1084	0.1080	0.1084	0.1082	0.1063	0.1059
	7	0.1081	0.1081	0.1081	0.1077	0.1061	0.1056
	7	0.1081	0.1078	0.1081	0.1069	0.1062	0.1058
set 2	7	0.1075	0.1072	0.1075	0.1070	0.1075	0.1070
	7	0.1091	0.1078	0.1091	0.1083	0.1091	0.1075
	7	0.1085	0.1076	0.1085	0.1074	0.1085	0.1083
set1	14	0.1078	0.1072	0.1078	0.1069	0.1037	0.1029
	14	0.1077	0.1070	0.1077	0.1073	0.1032	0.1026
	14	0.1081	0.1071	0.1081	0.1078	0.1034	0.1021
set 2	14	0.1067	0.1052	0.1067	0.1051	0.1067	0.1062
	14	0.1065	0.1048	0.1065	0.1062	0.1065	0.1051
	14	0.1066	0.1050	0.1066	0.1058	0.1066	0.1056
set1	28	0.1072	0.1056	0.1072	0.1074	0.1061	0.1056
	28	0.1068	0.1066	0.1068	0.1078	0.1058	0.1047
	28	0.1074	0.1069	0.1074	0.1079	0.1060	0.1051
set 2	28	0.1057	0.1055	0.1057	0.1055	0.1057	0.1057
	28	0.1062	0.1062	0.1062	0.1063	0.1062	0.1048
	28	0.1073	0.1050	0.1073	0.1054	0.1073	0.1054
set1	50	0.1093	0.1082	0.1093	0.1083	0.1041	0.1043
	50	0.1088	0.1082	0.1088	0.1091	0.1049	0.1041
	50	0.1086	0.1080	0.1086	0.1090	0.1043	0.1041
set 2	50	0.1076	0.1066	0.1076	0.1071	0.1076	0.1083
	50	0.1080	0.1078	0.1080	0.1077	0.1080	0.1067
	50	0.1084	0.1077	0.1084	0.1073	0.1084	0.1074

Table B6. The concentration of andrographolide (mg/ml) in Sample B

Sample B	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	0.1161	0.1164	0.1161	0.1164	0.1158	0.1136
	4	0.1163	0.1162	0.1163	0.1168	0.1146	0.1141
	4	0.1166	0.1171	0.1166	0.1147	0.1148	0.1136
set 2	4	0.1194	0.1196	0.1194	0.1193	0.1194	0.1192
	4	0.1200	0.1198	0.1200	0.1201	0.1200	0.1187
	4	0.1202	0.1204	0.1202	0.1201	0.1202	0.1192
set1	7	0.1125	0.1121	0.1125	0.1118	0.1155	0.1146
	7	0.1118	0.1127	0.1118	0.1111	0.1155	0.1141
	7	0.1114	0.1129	0.1114	0.1122	0.1146	0.1144
set 2	7	0.1228	0.1225	0.1228	0.1214	0.1228	0.1216
	7	0.1222	0.1224	0.1222	0.1213	0.1222	0.1206
	7	0.1219	0.1235	0.1219	0.1221	0.1219	0.1215
set1	14	0.1144	0.1146	0.1144	0.1142	0.1148	0.1143
	14	0.1142	0.1151	0.1142	0.1145	0.1162	0.1148
	14	0.1143	0.1139	0.1143	0.1138	0.1149	0.1144
set 2	14	0.1156	0.1161	0.1156	0.1167	0.1156	0.1162
	14	0.1168	0.1166	0.1168	0.1154	0.1168	0.1154
	14	0.1167	0.1160	0.1167	0.1164	0.1167	0.1152
set1	28	0.1132	0.1127	0.1132	0.1128	0.1174	0.1161
	28	0.1131	0.1124	0.1131	0.1128	0.1168	0.1149
	28	0.1128	0.1134	0.1128	0.1128	0.1160	0.1160
set 2	28	0.1169	0.1172	0.1169	0.1164	0.1169	0.1165
	28	0.1169	0.1165	0.1169	0.1160	0.1169	0.1163
	28	0.1169	0.1158	0.1169	0.1155	0.1169	0.1171
set1	50	0.1137	0.1141	0.1137	0.1151	0.1129	0.1135
	50	0.1151	0.1147	0.1151	0.1152	0.1140	0.1125
	50	0.1153	0.1145	0.1153	0.1140	0.1138	0.1122
set 2	50	0.1208	0.1206	0.1208	0.1192	0.1208	0.1211
	50	0.1208	0.1192	0.1208	0.1191	0.1208	0.1197
	50	0.1197	0.1198	0.1197	0.1202	0.1197	0.1201

Table B7. The concentration of andrographolide (mg/ml) in Sample C

Sample C	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	0.1664	0.1638	0.1576	0.1586	0.1669	0.1666
	4	0.1658	0.1662	0.1575	0.1570	0.1672	0.1667
	4	0.1669	0.1670	0.1586	0.1586	0.1667	0.1670
set 2	4	0.1664	0.1658	0.1576	0.1576	0.1669	0.1672
	4	0.1658	0.1667	0.1575	0.1579	0.1672	0.1664
	4	0.1669	0.1656	0.1586	0.1577	0.1667	0.1665
set1	7	0.1691	0.1678	0.1657	0.1653	0.1601	0.1600
	7	0.1694	0.1694	0.1659	0.1656	0.1609	0.1596
	7	0.1702	0.1701	0.1670	0.1660	0.1608	0.1606
set 2	7	0.1691	0.1686	0.1657	0.1656	0.1601	0.1587
	7	0.1694	0.1692	0.1659	0.1659	0.1609	0.1604
	7	0.1702	0.1688	0.1670	0.1652	0.1608	0.1601
set1	14	0.1700	0.1714	0.1646	0.1658	0.1598	0.1593
	14	0.1715	0.1704	0.1651	0.1656	0.1573	0.1581
	14	0.1711	0.1709	0.1669	0.1647	0.1584	0.1591
set 2	14	0.1700	0.1688	0.1646	0.1650	0.1598	0.1584
	14	0.1715	0.1688	0.1651	0.1652	0.1573	0.1595
	14	0.1711	0.1679	0.1669	0.1632	0.1584	0.1586
set1	28	0.1647	0.1651	0.1593	0.1603	0.1602	0.1591
	28	0.1650	0.1638	0.1592	0.1585	0.1594	0.1586
	28	0.1641	0.1641	0.1604	0.1602	0.1587	0.1588
set 2	28	0.1647	0.1646	0.1593	0.1594	0.1602	0.1587
	28	0.1650	0.1625	0.1592	0.1592	0.1594	0.1587
	28	0.1641	0.1640	0.1604	0.1599	0.1587	0.1589
set1	50	0.1652	0.1645	0.1605	0.1609	0.1551	0.1558
	50	0.1648	0.1652	0.1605	0.1602	0.1570	0.1548
	50	0.1646	0.1644	0.1607	0.1606	0.1581	0.1551
set 2	50	0.1652	0.1646	0.1605	0.1602	0.1551	0.1567
	50	0.1648	0.1641	0.1605	0.1594	0.1570	0.1548
	50	0.1646	0.1643	0.1607	0.1592	0.1581	0.1560

Table B8 Percentage of andrographolide quantity of sample A calculated from 15.0 grams of crude powder.

Sample A	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	1.4575	1.4526	1.4573	1.4490	1.4467	1.4352
	4	1.4537	1.4532	1.4533	1.4503	1.4453	1.4272
	4	1.4591	1.4363	1.4587	1.4475	1.4467	1.4416
set 2	4	1.4386	1.4265	1.4386	1.4257	1.4386	1.4305
	4	1.4163	1.4219	1.4163	1.4282	1.4163	1.4272
	4	1.4330	1.4142	1.4330	1.4143	1.4330	1.4125
set1	7	1.4453	1.4396	1.4453	1.4425	1.4173	1.4121
	7	1.4413	1.4253	1.4413	1.4362	1.4147	1.4084
	7	1.4413	1.4304	1.4413	1.4251	1.4160	1.4107
set 2	7	1.4329	1.4296	1.4329	1.4263	1.4329	1.4269
	7	1.4550	1.4379	1.4550	1.4437	1.4550	1.4331
	7	1.4465	1.4346	1.4465	1.4316	1.4465	1.4434
set1	14	1.4378	1.4250	1.4373	1.4249	1.3825	1.3716
	14	1.4367	1.4270	1.4360	1.4309	1.3754	1.3676
	14	1.4416	1.4284	1.4413	1.4377	1.3787	1.3617
set 2	14	1.4221	1.4027	1.4221	1.4018	1.4221	1.4154
	14	1.4197	1.3973	1.4197	1.4165	1.4197	1.4010
	14	1.4208	1.3997	1.4208	1.4107	1.4208	1.4079
set1	28	1.4293	1.4076	1.4298	1.4324	1.4152	1.4085
	28	1.4240	1.4220	1.4241	1.4367	1.4111	1.3956
	28	1.4320	1.4260	1.4324	1.4388	1.4134	1.4018
set 2	28	1.4096	1.4066	1.4096	1.4063	1.4096	1.4099
	28	1.4159	1.4153	1.4159	1.4169	1.4159	1.3970
	28	1.4309	1.4001	1.4309	1.4047	1.4309	1.4048
set1	50	1.4573	1.4421	1.4573	1.4443	1.3874	1.3905
	50	1.4507	1.4433	1.4507	1.4544	1.3987	1.3886
	50	1.4480	1.4404	1.4480	1.4533	1.3907	1.3884
set 2	50	1.4351	1.4215	1.4351	1.4286	1.4351	1.4439
	50	1.4397	1.4376	1.4397	1.4357	1.4397	1.4220
	50	1.4456	1.4360	1.4456	1.4309	1.4456	1.4316

Table B9 Percentage of andrographolide quantity of sample B calculated from 15.0 grams of crude powder.

Sample B	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	1.5485	1.5526	1.5485	1.5521	1.5444	1.5150
	4	1.5508	1.5499	1.5508	1.5570	1.5280	1.5215
	4	1.5548	1.5618	1.5548	1.5293	1.5302	1.5145
set 2	4	1.5921	1.5942	1.5921	1.5903	1.5921	1.5897
	4	1.6000	1.5971	1.6000	1.6017	1.6000	1.5833
	4	1.6022	1.6049	1.6022	1.6014	1.6022	1.5888
set1	7	1.4997	1.4952	1.4997	1.4904	1.5405	1.5281
	7	1.4911	1.5025	1.4911	1.4817	1.5395	1.5211
	7	1.4847	1.5053	1.4847	1.4962	1.5282	1.5254
set 2	7	1.6372	1.6338	1.6372	1.6189	1.6372	1.6213
	7	1.6295	1.6323	1.6295	1.6167	1.6295	1.6084
	7	1.6260	1.6470	1.6260	1.6276	1.6260	1.6201
set1	14	1.5251	1.5283	1.5251	1.5226	1.5307	1.5243
	14	1.5228	1.5341	1.5228	1.5262	1.5487	1.5302
	14	1.5237	1.5186	1.5237	1.5174	1.5326	1.5259
set 2	14	1.5414	1.5479	1.5414	1.5557	1.5414	1.5494
	14	1.5569	1.5547	1.5569	1.5389	1.5569	1.5388
	14	1.5560	1.5466	1.5560	1.5523	1.5560	1.5363
set1	28	1.5096	1.5021	1.5096	1.5043	1.5655	1.5478
	28	1.5085	1.4984	1.5085	1.5043	1.5577	1.5325
	28	1.5045	1.5115	1.5045	1.5044	1.5461	1.5465
set 2	28	1.5581	1.5632	1.5581	1.5523	1.5581	1.5530
	28	1.5586	1.5531	1.5586	1.5464	1.5586	1.5504
	28	1.5588	1.5442	1.5588	1.5395	1.5588	1.5613
set1	50	1.5159	1.5212	1.5159	1.5341	1.5058	1.5129
	50	1.5349	1.5298	1.5349	1.5365	1.5194	1.5005
	50	1.5370	1.5269	1.5370	1.5203	1.5175	1.4961
set 2	50	1.6113	1.6074	1.6113	1.5890	1.6113	1.6143
	50	1.6110	1.5888	1.6110	1.5885	1.6110	1.5961
	50	1.5957	1.5969	1.5957	1.6033	1.5957	1.6016

Table B10 Percentage of andrographolide quantity of sample C calculated from 15.0 grams of crude powder.

Sample C	No. of day	irradiated 5 kGy		irradiated 10 kGy		irradiated 25 kGy	
		control	sample	control	sample	control	sample
set1	4	2.2191	2.1842	2.1009	2.1152	2.2258	2.1961
	4	2.2112	2.2157	2.0995	2.0932	2.2289	2.2094
	4	2.2249	2.2262	2.1151	2.1145	2.2232	2.1874
set 2	4	2.2191	2.2109	2.1009	2.1010	2.2258	2.2287
	4	2.2112	2.2226	2.0995	2.1059	2.2289	2.1794
	4	2.2249	2.2075	2.1151	2.1021	2.2232	2.2038
set1	7	2.2551	2.2377	2.2089	2.2034	2.1342	2.1334
	7	2.2582	2.2584	2.2117	2.2080	2.1449	2.1281
	7	2.2695	2.2678	2.2269	2.2134	2.1436	2.1411
set 2	7	2.2551	2.2475	2.2089	2.2084	2.1342	2.1155
	7	2.2582	2.2556	2.2117	2.1998	2.1449	2.1385
	7	2.2695	2.2505	2.2269	2.1999	2.1436	2.1340
set1	14	2.2673	2.2854	2.1951	2.2106	2.1306	2.1245
	14	2.2862	2.2719	2.2019	2.2083	2.0967	2.1085
	14	2.2807	2.2787	2.2247	2.1964	2.1119	2.1217
set 2	14	2.2673	2.2506	2.1951	2.2006	2.1306	2.1120
	14	2.2862	2.2510	2.2019	2.2030	2.0967	2.1264
	14	2.2807	2.2382	2.2247	2.1766	2.1119	2.1151
set1	28	2.1957	2.2009	2.1241	2.1372	2.1358	2.0889
	28	2.2005	2.1834	2.1229	2.1131	2.1258	2.1017
	28	2.1885	2.1886	2.1383	2.1362	2.1160	2.1076
set 2	28	2.1957	2.1950	2.1241	2.1254	2.1358	2.1100
	28	2.2005	2.1667	2.1229	2.1224	2.1258	2.1000
	28	2.1885	2.1861	2.1383	2.1315	2.1160	2.1125
set1	50	2.2024	2.1937	2.1405	2.1289	2.0682	2.0778
	50	2.1979	2.2025	2.1526	2.1366	2.0928	2.0640
	50	2.2125	2.1915	2.1425	2.1094	2.1086	2.0686
set 2	50	2.2024	2.1812	2.1405	2.1029	2.0682	2.0891
	50	2.1979	2.1881	2.1526	2.1248	2.0928	2.0639
	50	2.2125	2.1907	2.1425	2.1232	2.1086	2.0806

Precision of measurement

Table B11 Precision of this experiment by measuring intra-batch and inter-batch variations.

Intra batch	Peak area	Conc.(mg/ml)	%Andrographolide
Precise A1	8425.207	0.1034	1.3785
	8451.836	0.1037	1.3829
	8411.116	0.1032	1.3762
Precise A2	8336.151	0.1023	1.3639
	8336.426	0.1023	1.3640
	8310.189	0.1020	1.3597
Precise A3	8421.672	0.1033	1.3779
	8366.928	0.1027	1.3690
	8395.733	0.1030	1.3737
Precise A4	8490.879	0.1042	1.3893
	8455.988	0.1038	1.3835
	8477.58	0.1040	1.3871
Precise A5	8424.742	0.1034	1.3784
	8363.126	0.1026	1.3683
	8394.642	0.1030	1.3735
Average	8404.148	0.1031	1.3751
SD	53.6967	0.0007	0.0088
%CV	-	-	0.64

Inter batch	Peak area	Conc. (mg/ml)	%Andrographolide
Precise B	8981.319	0.1102	1.4695
	8992.905	0.1104	1.4714
	8942.361	0.1097	1.4631
Precise C	9476.278	0.1163	1.5505
	9393.689	0.1153	1.5370
	9463.717	0.1161	1.5484
Precise D	9493.009	0.1165	1.5532
	9400.114	0.1154	1.5380
	9428.868	0.1157	1.5427
Average	9285.807	0.1140	1.5193
SD	237.827	0.0029	0.0389
%CV	-	-	2.56

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